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# STUDIES ON GALL BLADDER FUNCTION

## VIII. THE FATE OF BILE PIGMENT AND CHOLESTEROL IN HEPATIC BILE SUBJECTED TO GALL BLADDER ACTIVITY

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Eleven years have passed since Rous and McMaster (15) published their paper on the concentrating activity of the gall bladder. This contribution and subsequent ones by them and their associates renewed the interest of investigators in this organ. Two schools have developed, each with a different fundamental concept of gall bladder function. The one, following what would appear to be the more logical concept, maintains that concentrated bile is emptied at intervals into the duodenum. The other, largely from teleological reasoning, believes that the cystic duct is a one-way tube, permitting hepatic bile to flow into the gall bladder, but preventing it from flowing out. Those who accept the latter concept must necessarily adhere to the theory that every constituent of the bile is absorbed through the gall bladder wall. If the latter group is correct there must be an optimum concentration for different constituents of the bile at which level they are absorbed at a definite rate.

The experiments of Rous and McMaster indicated that bile pigment was not absorbed through the gall bladder wall. If this is true, and if the gall bladder has no other way of evacuating itself except by absorption, it would not take long for the lumen to be filled with an inspissated mass of pigment. The experiments on the pigment changes of hepatic bile subjected to gall bladder activity were open to certain errors which Rous and McMaster duly considered. The experiments were not absolutely quantitative in that the amount of hepatic bile entering the gall bladder could not be absolutely measured.

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It was thought that a modification of their method might offer additional evidence on the fate of bilirubin in the gall bladder.

There has been considerable controversy in the literature on gall bladder function as to whether cholesterol is absorbed from, secreted into, or merely concentrated in gall bladder bile. The polemic is an old one, having been carried on for years by Aschoff (2) and Naunyn (12). The literature on this subject has been fully reviewed (6, 9, 10). Data on this controversial point are important because of the relationship which cholesterol bears to calculus formation. Recent papers published by Elman and Taussig (5, 6), and Elman and Graham (4) support the Naunyn hypothesis in that these workers present evidence which would appear to show that the normal gall bladder secretes cholesterol into the gall bladder bile.

Data which we have accumulated do not agree with this point of view. The differences should be easily reconciled if a method can be devised which will permit quantitative studies in a normally functioning gall bladder. We have recently described such a method (13). It permits quantitative studies in a gall bladder which is free of any ductal connection with the liver, but which retains its normal blood supply and major lymphatic vessels. The animals are normal in that a large proportion of the bile from the liver still passes into the intestine. The appetite is unaltered and nutrition does not suffer.

### *Method*

The method of intubation of the gall bladder has been sufficiently described (13). It consists in isolating the gall bladder from any ductal connection with the liver and inserting a soft Nélaton catheter through a slit in the hepatic duct just above the entrance of the right and left lobe ducts. The catheter is passed through the cystic duct to a position just inside the lumen of the gall bladder. When it is *in situ* a ligature is passed around the common hepatic duct and tied so as to hold the catheter in position. The catheter is brought out through a stab wound to either side of the incision.

The animals remain in excellent condition after operation.<sup>1</sup> They are dressed with the same care used during the operation in order to maintain asepsis.

This method has been used in this laboratory for over 2 years during which period we have been convinced that the preliminary operative

<sup>1</sup> The operations were done under ether anesthesia.

procedure does not alter normal function. Repeated injection of the lymphatics after this procedure has shown that the major lymphatic drainage from the gall bladder is not disturbed. The cystic artery and vein are never damaged. The gall bladder is not handled during the operation. Every visible accessory duct is ligated if this can be done without damage to the gall bladder lymphatics or blood vessels; otherwise the animals are discarded. The studies on bile have all been made on the unanesthetized animal.

Every preparation is tested for the presence of unligated accessory ducts and for the ability of the gall bladder to absorb water by placing a known amount of normal sodium chloride in the gall bladder after having cleansed it and permitting the solution to remain for from 1 to 3 hours. If accessory ducts have been overlooked, the clear solution becomes bile-tinged. Furthermore, every animal has been carefully autopsied after the injection of methylene blue into the gall bladder. This serves as an added check on the presence of accessory ducts. These are so frequent in the dog that any experiment which fails to consider their presence is of doubtful value. We have recently completed anion-cation studies of hepatic bile subjected to gall bladder activity and have found that the organ as prepared by our method will alter the hepatic bile in composition so that the bile removed is nearly identical with the gall bladder bile removed from the organ at the time of intubation.

The hepatic bile was obtained from cholecystectomized dogs after double catheterization of the common duct (14). Since the bile was never collected for longer than 24 hours before it was again turned into the duodenum, the animals remained in excellent condition.

Cultures and smears were made constantly of the bile of the animals of each group and, as soon as infection occurred, they were discarded. Rigid asepsis is necessary in any experiment of this type.

The bile pigment was estimated by the Hooper and Whipple method (7) with the modification of the inorganic standard as suggested by Rous and McMaster (15). A series of standards was found advantageous for close comparison with the unknowns. Furthermore, since nearly all of the determinations were made during the summer months, it was necessary to keep the tubes at a constant temperature for a definite period after the introduction of the acid alcohol. Controls run at the beginning of the experiment showed us that the maximum depth of the bluish green color developed after 4 hours at 38°C. This time and temperature were therefore adopted for all determinations. Merely waiting until the color turns green, as recently suggested (6), results in inaccurate estimations of the pigment present. The color turns bluish green long before the maximum intensity is developed. Occasionally the color becomes purplish or grayish so that comparisons are not possible. Using known amounts of pigment, the error of the Hooper and Whipple method has been about 5 per cent in our hands.

All bile pigment readings were made with a monochromatic neon lamp provided with a Corning filter. The neon tube was 32 inches long of  $\frac{1}{4}$  inch Pyrex tubing doubled on itself eight times. The filters were heat-resisting, red, 3.95 mm. thick, and heat-absorbing, 2.95 mm. thick, of medium shade.

The cholesterol was determined by the method of Autenrieth and Funk, as described by McMaster (11) with readings against a known cholesterol standard. The error in the determination of known amounts in our hands has been from 10 to 15 per cent. The method is far from ideal.

## RESULTS

*Bile Pigment.*—Known amounts of hepatic bile of a determined bile pigment concentration were introduced into the gall bladder and permitted to remain there for from 2 to 24 hours. In some of the experiments further additions of hepatic bile were made from time to time so that the total amount of hepatic bile received by the gall bladder was frequently several times that which the organ originally held. When the bile was withdrawn, its amount was carefully determined and the gall bladder was then washed out twice with normal saline. The concentration of the pigment in the specimen and in the washings was then determined and the two added in order to find the total amount recovered.

Table I shows the changes in fluid content after hepatic bile was placed in the gall bladder. These vary considerably because of difference in the time when the last hepatic bile was introduced. From 64 to 93.4 per cent of the introduced fluid was removed in a 24 hour period.

It is evident that in those instances in which the amount of pigment withdrawn exceeded the amount introduced the increase was small and was within the error of the method. In sixteen of the eighteen experiments there was a small apparent loss of bile pigment. In only three instances (Dogs 521, 44, and 45) did the amount lost exceed the error of the method and then only very slightly. In these experiments the fluid change was from 26.5 to 2.5 cc., 32.5 to 7 cc., and from 25 to 4.5 cc. respectively. The pigment loss was therefore relatively small in comparison to the fluid loss of 90.6, 81, and 86 per cent.

Table II gives the results of eleven experiments in which hepatic bile was placed in the bile-free gall bladder and cholesterol determinations made. The cholesterol concentrations of the introduced material

varied from 11 mg. per 100 cc. to 46 mg. per 100 cc. of bile. These concentrations are all below those of cholesterol in dogs' blood. The concentrations in the bile removed from the gall bladder after the

TABLE I  
*Bile Pigment Changes in Hepatic Bile Subjected to Gall Bladder Activity*

Dog No.	Time	Concentration in	Concentration out	Volume in	Volume out	Amount in	Amount out	Amount change	Change
	hrs.	gm./100 cc.	gm./100 cc.	cc.	cc.	mg.	mg.	mg.	per cent
1134	2	1.61	5.29	18.5	5.0	28.98	26.45	-2.53	-8.7
1134	18	1.70	9.16	18.0	2.5	30.60	27.60	-3.00	-9.8
1134	2	0.39	0.53	15.0	11.0	5.85	5.83	-0.02	-0.3
1134	2	0.55	0.56	13.0	11.5	7.15	7.13	-0.02	-0.2
1134	16	0.32	2.59	13.0	1.5	4.16	3.89	-0.27	-6.4
1119	2	0.35	0.76	20.0	6.5	7.00	6.29	-0.71	-10.1
15	22	1.80	—	15.0	1.0	27.00	24.43	-2.57	-9.5
521	24	1.02	—	26.5	2.5	27.03	23.80	-3.23	-11.9
44	24	0.56	1.90	32.5	7.0	18.20	15.90	-2.30	-12.6
40	24	0.39	1.86	18.5	3.5	7.22	7.53	+0.31	+4.2
45	24	0.83	3.00	25.0	4.5	20.75	18.06	-2.69	-12.9
6	24	1.20	3.70	35.0	9.7	42.00	39.99	-2.01	-4.7
6	2	2.20	2.10	20.0	17.5	44.00	44.49	+0.49	+1.1
60	24	0.82	2.70	18.0	4.5	14.76	14.07	-0.69	-4.6
184	7	0.86	1.20	35.0	24.0	30.10	29.80	-0.30	-0.9
184	17	1.02	1.50	40.0	26.0	40.80	39.60	-1.20	-2.9
186	12	0.38	0.66	20.0	9.0	7.60	7.49	-0.11	-1.4
186	2	0.32	0.49	20.0	12.5	6.40	6.73	-0.33	-4.9
Mean.....									-5.36
Median.....									-4.8

hepatic bile had been subjected to gall bladder activity for a variable period ranged from 25 to 141 mg. per 100 cc., the latter approaching the blood level. In only one experiment in which the gall bladder was

washed was there an increase in the total amount of cholesterol removed over the amount introduced, and this was well within the error of the method. The mean difference between the amount of cholesterol introduced and the amount removed at the end of the experiment was 10.5 per cent. In every experiment except one the percentage removal of fluid was considerably greater than the percentage loss of cholesterol. In this experiment, Dog 65, the bile placed in the gall

TABLE II  
*Cholesterol Changes in Hepatic Bile in the Gall Bladder*

Dog No.	Time	Concentration in	Concentration out	Volume in	Volume out	Amount in	Amount out	Amount change	Change
	hrs.	gm./100 cc.	gm./100 cc.	cc.	cc.	mg.	mg.	mg.	per cent
702	30	0.046	0.141	49.0	15.5	22.5	23.5	+1.0	+ 4.4
642	21½	0.026	0.121	50.5	6.8	13.1	11.3	-1.8	-13.7
642	24	0.026	0.062	33.0	8.0	8.6	6.8	-1.8	-20.9
737	24	0.026	0.087	35.0	8.5	9.1	8.4	-0.7	-7.6
190	6	0.019	0.031	40.0	16.0	7.6	5.0	-2.6	-12.7*
190	12	0.016	0.050	60.0	20.0	9.6	10.0	+0.4	
218	5	0.027	0.096	20.0	7.5	5.4	7.2	+1.8	-18.1*
218	12½	0.027	0.140	20.0	3.0	5.4	4.2	-1.2	
218	7	0.026	0.029	20.0	6.0	5.2	1.7	-3.5	
65	24	0.123	0.124	10.0	9.5	12.3	11.8	-0.5	-4.0†
1123	24	0.011	0.025	48.75	19.0	5.4	4.8	-0.6	-11.1

\* No washings.

† Gall bladder bile.

bladder was the gall bladder bile from another dog. The loss of fluid in this experiment was quite small, as would be expected.

In order to show the error which may arise if accessory ducts are not carefully excluded, Table III is presented. In every instance tabulated, one or more accessory ducts existed which were not seen at operation. Some of these entered the gall bladder directly while others emptied into various portions of the cystic duct. Thus hepatic bile was being added to the gall bladder over and above the amount

which we were introducing artificially. The concentrations of cholesterol in the bile introduced varied from 12.0 to 26.0 mg. per 100 cc., while that of the bile removed from the gall bladder varied from 35.0 to 150 mg. per 100 cc. In every instance there was an increase in the total amount of cholesterol removed, although in every other respect (loss of fluid, concentration, etc.) the results were quite similar to those obtained from animals whose gall bladders had no accessory ducts. The increase in the total amount of cholesterol was above the amount which could be explained by the error of the method of determination.

That infection of the gall bladder may cause the cholesterol recovered to be greater in amount than that introduced is shown in the following

TABLE III  
*Cholesterol—Dogs with Accessory Ducts*

Dog No.	Time	Concen- tration in	Concen- tration out	Volume in	Volume out	Amount in	Amount out	Amount change	Change
	hrs.	gm./100 cc.	gm./100 cc.	cc.	cc.	mg.	mg.	mg.	per cent
610	21	0.012	0.082	40.0	7.3	4.8	6.9	2.3	+47.9
610	48	0.012	0.082	56.0	10.5	6.7	8.6	1.9	+28.3
639	30	0.020	0.150	56.0	13.5	11.2	23.7	12.5	+111.6
640	17	0.026	0.132	20.0	2.3	5.2	6.2	1.0	+19.2
640	24	0.026	0.055	40.0	26.0	10.4	14.3	3.9	+37.5

experiment:—Dog 190. After infection of the gall bladder wall we obtained a 96 per cent increase in the total cholesterol in a 24 hour period. The total amount of cholesterol introduced was 12 mg. while the total amount removed was 23.5 mg.

#### DISCUSSION

As already stated, studies in this laboratory have shown us that the type of preparation we are using provides a normal gall bladder. Hunt, Davis, and Boyden (8) have shown that ligation of the cystic duct or tying the cystic mesentery in such a way as to produce stasis in the gall bladder, even though the arterial circulation is not interrupted, results in cholecystitis. The changes which they found

ranged from the mildest inflammation to hydropic distention of the gall bladder.

The disappearance of bile pigment during an inflammatory reaction of the gall bladder wall cannot be taken as evidence that, under normal conditions, the same thing will occur. In Dog 1119, after infection, 33.8 per cent of the bile pigment was lost from the gall bladder in a 2.5 hour period and, in Dog 17, 36 per cent of pigment was lost in a 2 hour period. We do not know whether this loss is due to absorption of the pigment or to a change in its composition making quantitative determination impossible.

Several facts are obvious from an analysis of our bile pigment data. If the bile is merely removed from the gall bladder without rinsing the organ, the amount of pigment recovered may be considerably lower than one would expect from the percentage of fluid lost from the gall bladder. In fact the pigment concentration may appear to be but slightly changed from that of the bile introduced. The amount recovered in the washings makes plain the fact that pigment clings to the gall bladder wall. Since the gall bladders used in these studies were carefully cleansed before the introduction of the hepatic bile, the pigment attached to the wall at the conclusion of the experiment could only have come from the bile introduced. A considerable amount of this can be removed by repeated aspiration and reinjection of the bile, as was done in Dog 1134, but the results vary. Therefore we finally adopted the method of merely aspirating the contained bile with a Luer syringe and subsequently washing the gall bladder with a measured amount of saline.

Undoubtedly the low pigment concentrations which are often reported for gall bladder bile are the result of a failure to recover the total pigment present. It is impossible, even by the method which we used, to recover quite all the pigment introduced since washings subsequent to those used in the final determination give dilutions so low as to make analyses untrustworthy. The mean loss of pigment in the eighteen experiments was 5.36 per cent and the median was 4.8 per cent.

We do not believe that one can assume from the data presented that any pigment is absorbed. Furthermore, we have repeatedly cannulated the cystic lymph vessels as did Rous and McMaster (15) and

have failed to demonstrate the presence of any bile pigment in the lymph from the gall bladder wall.

A difference of opinion still exists as to whether cholesterol is absorbed by the gall bladder mucosa (Aschoff), or is secreted by it (Naunyn). Among the more recent work supporting the absorption theory may be mentioned that of Torinoumi (17), Illingworth (9), Boyd (3), and Andrews, Schoenheimer, and Hrdina (1). Boyd (3) did not actually measure the cholesterol content of gall bladder bile, but drew his conclusions from histologic studies and from changes in the cholesterol content of the blood after cholecystectomy. Illingworth (9) concluded from experiments on two cats that when cholesterol is present in excess it can be absorbed. He gave no data on the amount of fluid in the gall bladder at the time of autopsy. The amount which may have precipitated out of the emulsion was not considered. If the contents increased considerably during the period of study it is likely that the gall bladder was not normal.

Torinoumi (17) found that cholesterol was absorbed from the normal gall bladder and secreted into the infected gall bladder. Andrews, Schoenheimer, and Hrdina (1) agree with these findings, although they have published no data to prove absorption in the normal animal. Elman and Taussig (5) found that, after ligation of the cystic duct, both the concentration and the total amount of cholesterol in the gall bladder were increased as a rule.

Elman and Taussig (6) and Elman and Graham (4) have more recently reported cholesterol analyses of hepatic and gall bladder bile from the same animal. The method which they used for estimating the amount of bile flowing into the gall bladder was the partitioning ligature method used by Rous and McMaster (15). This method is not an exact quantitative method since the amount of bile secreted per gram of liver tissue may not be the same under the conditions of the experiment. Furthermore, no mention is made of the exclusion of accessory biliary ducts. Failure to eliminate these may give results similar to those which Elman and his associates have presented for apparently normal animals.

While Rous and McMaster (15) have shown that bile pigment under normal conditions is excreted in nearly uniform concentrations from different portions of the liver, there are no data upon which one can base the assumption that every other constituent of the bile is similarly excreted. Furthermore, the experiments of Elman and Taussig (6) which use bile pigment as a measure of gall bladder activity fail to show concentration of bile pigment in two of the four experiments, a condition we have never encountered in the normally functioning gall bladder of the dog.

From histologic studies made in this laboratory, and from the observations of Hunt, Davis, and Boyden (8) it would appear useless



to consider as normal the cholesterol figures obtained from animals whose cystic duct has been ligated. It would seem equally useless to discuss any data in which the numerous accessory ducts entering the cystic duct or gall bladder had not been taken into account in estimating the total bile received by the gall bladder.

The increases in cholesterol content which Elman and Taussig (6) and Elman and Graham (4) have found in human gall bladder bile were from obviously diseased gall bladders. That cholesterol may increase when infection or inflammation is present cannot be doubted. The data presented from Dog 190 after infection had occurred agree with their findings. One cannot assume, however, that the gall bladder membrane acts in exactly the same manner when normal and when diseased.

#### SUMMARY

The data obtained from the experiments on dogs reported in this paper lead us to conclude that bile pigment is not absorbed from the gall bladder bile. The mean loss of pigment is so small when compared with the amount of water lost that it is negligible.

Our cholesterol data do not support the concept that this substance is secreted into the gall bladder bile of the dog under normal conditions. In the majority of experiments there was a loss of cholesterol. Indeed we have failed to find any evidence of definite secretion or absorption save in the case of the infected gall bladder. We are led to conclude, as did Rous and McMaster (15) with regard to bile pigment, that normally there is no absorption of cholesterol.

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## STUDIES ON VIR

### IV. INFLUENCE ON VIRULENCE OF PNEUMOCOCCI OF GROWTH ON VARIOUS MEDIA\*

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During a series of investigations of some of the factors influencing virulence of microorganisms grown *in vitro*, the effect of the material used for medium was naturally of prime importance. As indicated in an earlier publication (1), there is much evidence in support of the view that virulence is correlated with the ability of a microorganism to grow in or on living animal tissue. Indeed, the best known method for increasing this microbic characteristic is animal passage. Furthermore, there is a well recognized predilection of a given microorganism for a certain tissue of the host, and also a variation in the influence of different organ extracts on growth *in vitro*. Consequently it seemed worth while to study the virulence of microorganisms grown on extracts of different organs of the animal body.

Extracts from many of the organs of the body have been studied as media for the growth of various organisms; but few investigators have reported on the effect of these media on virulence. Thus, in more recent literature, Graham-Smith (2), in 1920, reported, in his study of conditions of growth, that staphylococcus and *B. coli* show a markedly better growth on ox pancreas extract than on meat extract. Proca (3), in 1924, made simple extracts from both spleen and liver of the calf, and found them satisfactory for growth of some ten organisms. In 1927, Hach (4) found that macerated brain substance (human or ox), although causing slight retardation in growth in about 700 strains studied, did not alter virulence of his strains of *B. anthracis*, *B. typhi murium*, *B. bipolaris septicus*, or *Staph. aureus*, even in periods ranging from 1 to 4 years. With a beef liver extract, Quiroga (5) (1928) has been able to maintain pneumococci at a high degree of virulence.

\* This is one of a series of studies carried out in part under a grant by the Influenza Commission of the Metropolitan Life Insurance Company.

Duran-Reynals (6) (1929), studying the effect of various organ extracts on vaccine virus, reported that as judged by the extent of lesions, testicle, kidney, and skin, and probably liver, brain, and placenta enhance the activity of the virus in rabbits. Pana (7) (1930) found that adrenalin or the suprarenal gland had no effect on the growth of *B. coli*, but that extracts of hypophysis, muscle, thymus, testicle, and thyroid increased the growth in this order. Applying the results of Duran-Reynals, Pijoan (8) (1931) tested the effect of testicle, kidney, and spleen extracts on twenty bacterial species, and as judged by the extent of the lesion produced, reported definite enhancement by testicular extract, slight enhancement by kidney, but lesions less than ordinary with spleen, at least when the organism used was staphylococcus.

### *Methods of Study*

In our previous work (9) in which an automatic device was used to make transfers every 2 to 4 hours, it was found that some media, not suitable for maintenance of virulence under ordinary transfer methods, would maintain this characteristic in this apparatus. Thus milk as a medium was observed to maintain or even to increase the virulence of some strains of pneumococci grown in the automatic transfer apparatus. Since conditions of growth supplied by the automatic transfer device differ from those of ordinary methods of transfer and perhaps more nearly simulate those found in the animal body, the possibility is suggested that the influence of the medium on the virulence of the organism might be better established in this apparatus.

It might be mentioned here that recently two other workers have described apparatus for continuous growth of organisms. A device similar to the one used in our experiments, described by Weiner (10), in 1927, was used by Friedlander and Meyer (11), and Friedlander and Hertert (12) in a study of the virulence of *B. aertrycke*. No increase of the virulence of this organism was found in 3 or 6 hour periods of transfer, for either mice or guinea pigs; but the authors suggested the possibility that this result may have been obtained because the organism was of maximum virulence at the beginning of the experiment. Also an apparatus, which was essentially a specially constructed, slow-running filter was suggested by Haddon (13) (1928) for continuous growth; but results of the use of this apparatus have not been found in the literature.

The present study is a report of the effect on virulence of growth of pneumococci in an automatic transfer apparatus on media made from different organ extracts.

The various media were made by the usual bacteriological method; that is, the organs from freshly killed animals were comminuted in a meat grinder, and infused overnight in the ice box in distilled water at the rate of 1 liter of water to 2 pounds of ground organs. In the morning, the infusion was boiled for from 15 to 30 minutes, and while hot the meat particles were strained out. The water extract was then titrated to pH 7.6, and sodium chloride added to make an additional 0.5 per cent. The neutralized medium in 5 liter Florence flasks was then sterilized in the autoclave at 17 pounds pressure for 1 hour. With one exception no peptone or other ingredients were added to the media reported in this study. As in earlier work, the virulence of pneumococci was estimated from the death of mice injected with dilutions of culture made in logarithmic series. The number of organisms per cubic centimeter was estimated by plating three dilutions of each culture on rabbit blood agar plates.

### *Calf Lung Medium*

This experiment is one of five in which calf lung medium was tested as to its suitability for maintenance of virulence of the pneumococcus.

TABLE I  
*Calf Lung Medium*  
2 hour interval of transfer

No. of transfers	No. of organisms per cc.	No. of organisms constituting fatal dose
168	40,000,000	4
432	150,000,000	15
600	120,000,000	12
696	48,000,000	4

A Neufeld Type I pneumococcus, of virulence such that one organism was fatal for a white mouse, was used to inoculate the lung medium in a machine regulated so that transfer was made every 2 hours. From preliminary experiments it was found that this medium was satisfactory for maintenance of a high degree of virulence over a considerable period of time. For that reason, the experiment recorded in Table I includes tests for virulence begun only after the 168th transfer. Briefly, it was found that, although the number of organisms in the culture receptacle varied considerably from time to time, the original virulence remained unchanged even up to the 696th transfer, or the equivalent of almost 2 years of daily reinoculation.

*Calf Heart Medium*

This experiment is similar to the previous one except that the medium was made from heart, instead of lung, of the calf. As in the case of lung, preliminary experiments indicated that heart medium supplied the necessary constituents for maintenance of virulence over short periods of time. Hence, in the experiment in Table II, the first estimation was made only after 72 transfers at 2 hour intervals. The average number of organisms per cubic centimeter in the growth receptable was higher in heart than was observed in lung medium. Since virulence tests in this experiment were run in dilutions no higher than 1-10,000,000, the end-point may not have been reached. It

TABLE II

*Calf Heart Medium*

2 hour interval of transfer

No. of transfers	No. of organisms per cc.	No. of organisms constituting fatal dose
72	120,000,000	120
192	50,000,000	50
360	240,000,000	24
432	230,000,000	23
504	250,000,000	25

is, however, quite evident that even after 504 transfers, the organism retained a high, and in all probability its maximum, virulence.

*Calf Spleen Medium*

Under ordinary methods of transfer spleen infusion proved to be unsatisfactory for maintenance of growth of pneumococci. However, the organisms were found to multiply on this medium when inoculated in the apparatus which made periodic transfers of culture at short intervals. To demonstrate the effect on virulence, two experiments are reported (Table III), the one on undiluted spleen medium and the second on spleen medium diluted with equal parts of physiological salt solution. When undiluted medium was used, a 4 hour period of transfer was found necessary to permit growth. In the diluted medium, however, a 2 hour interval was sufficient. The outstanding

differences between the growth on this organ extract and that on heart or lung extract were first, depression of the rate of multiplication of the organism, and second, rapid decrease in virulence. These differences are particularly well brought out in undiluted spleen medium. However, even with diluted medium, although virulence remained maximum through 168 transfers, there was subsequently a gradual decrease until 1 cc. of the culture containing 16 million microorganisms, was necessary to produce a fatal infection. The difference between the diluted and undiluted media would indicate that there may be some substance in the spleen which causes a decrease of virulence at least of

TABLE III  
*Calf Spleen Medium*

No. of transfers	No. of organisms per cc.	No. of organisms constituting fatal dose
Undiluted medium—4 hour interval of transfer		
42	25,000,000	2,500
78	20,000,000	200,000
120	10,000,000	10,000,000
Diluted medium—2 hour interval of transfer		
168	11,500,000	11
240	280,000,000	280
336	100,000,000	10,000
600	16,000,000	16,000,000

Type I pneumococcus. Whereas the diluted medium produced better growth and maintained virulence over a relatively longer period, the undiluted caused a rapid attenuation. This finding is thus seen to parallel the results of the work of Duran-Reynals and of Pijoan that spleen extracts depress the activity of vaccine virus and the virulence of staphylococcus.

#### *Horse Skeletal Muscle Medium*

In previous work, calf skeletal muscle proved to be an unsatisfactory medium for maintenance of virulent pneumococci; an infusion without peptone caused a very rapid decrease. Addition of peptone



or dextrose somewhat improved the quality of the medium, but in no experiment was it possible to make a medium from this extract which was capable of maintaining virulence. In the present experiment, skeletal muscle of the horse was used to determine the difference between the tissue from this animal and the one from the calf. As in most of the experiments with calf skeletal muscle, the medium in this experiment contained 1 per cent peptone. As can be seen from Table IV, this extract not only supported a rapid growth but also maintained a high degree of virulence over the period represented by 258 transfers. It would appear thus that tissues of the same type from two animal species contain, or lack perhaps, substances which cause the difference in activity of these extracts. For both

TABLE IV  
*Horse Skeletal Muscle Medium*  
2 hour interval of transfer

No. of transfers	No. of organisms per cc.	No. of organisms constituting fatal dose
54	44,000,000	4
102	140,000,000	14
144	240,000,000	24
210	200,000,000	20
258	124,000,000	12

support growth equally; yet growth on the one, calf skeletal muscle, results in a decrease of virulence, while growth on the other, horse muscle, causes no alteration of this property.

*Normal and Immune Horse Sera as Media*

Serum from normal animals has been found by many investigators to maintain virulence of various microorganisms over at least a short period of time. Because of the observations of Stryker (14), confirmed by other investigators, that antipneumococcus horse serum supports the growth of pneumococcus but with a resultant rapid decrease in virulence, this experiment was planned to ascertain whether growth *in vitro* in the automatic transfer device would give similar results. This experiment consequently is a report of the difference in results obtained by the growth of pneumococci on normal horse serum and on

the serum of the same horse after immunization against Type I pneumococcus. The results in Table V show that the serum of the horse before immunization was a suitable medium for at least 90 transfers. On the other hand, the serum from the same horse after immunization caused a drop of virulence approximately 250 thousand-fold, and in 36 transfers 40 million-fold. In other words, the experiment confirms the observations of Stryker that the pneumococcus, although multiplying at a normal rate in immune serum, had lost its pathogenicity. This was found true even when the immune serum was highly diluted with normal horse serum. For in one experiment, in which immune

TABLE V  
*Normal and Immune Horse Serum*

4 hour interval of transfer		
No. of transfers	No. of organisms per cc.	No. of organisms constituting fatal dose
<i>Before immunization</i>		
18	200,000,000	200
60	85,000,000	8
90	93,000,000	14
<i>After immunization</i>		
18	25,000,000	250,000
36	40,000,000	40,000,000
54	120,000,000	120,000,000

serum was diluted with 500 volumes of normal horse serum, the virulence decreased in 60 transfers over 100 thousand-fold. Such striking differences between the effect of immune horse serum and that of normal serum led to an analysis of immune serum with the object of finding the fraction which caused this decrease in virulence. It was observed that the supernatant serum, after eliminating a water-insoluble protein fraction obtained by dilution of immune serum with ten volumes of water (15), supported growth and when transferred at 4-hour intervals maintained maximum virulence of pneumococci for at least 60 transfers. On the other hand, a salt solution of the water-insoluble fraction to which 0.1 per cent peptone was added supported

growth of pneumococcus, but, as in the case of the whole immune serum, caused a sudden drop in virulence. The water-insoluble fraction of the antipneumococcus serum was found to contain all the so called antibodies of pneumococcus. It would appear therefore, that the pneumococcus antibody is in the fraction of immune serum which causes the decrease in virulence.

### *Rabbit and Guinea Pig Media*

In addition to the study of organ tissue extracts, two experiments are included on the extracts of two animal species, rabbit and guinea

TABLE VI, *a*  
*Whole Rabbit Medium*

No. of transfers	No. of organisms per cc.	No. of organisms constituting fatal dose
2 hour interval		
36	240,000,000	24
84	100,000,000	10
180	8,000,000	8,000
240	50,000,000	50,000
4 hour interval		
60	60,000,000	6,000
102	50,000,000	5,000
150	20,000,000	2,000
198	20,000,000	2,000,000

pig. These were chosen because of their difference in susceptibility to pneumococcus infection: the rabbit being highly susceptible, the guinea pig less so. The technic in preparing the media here used was similar to that given above, the only difference being that the entire animal including skin and bones, except the intestinal tract, was ground and infused. Table VI, *a*, represents the results obtained in the case of the rabbit medium with 2 and 4 hour intervals of transfer. With the 2 hour interval the virulence remained high for 84 transfers, then decreased gradually to an avirulent state. The longer period of transfer showed a lower rate of multiplication and a more rapid decrease in virulence than the 2 hour period.

Several experiments have been run with guinea pig medium, and in all, the results have been found to be similar to those represented in Table VI, *b*. No experiment was carried out, however, for a time longer than that represented by 144 transfers. The striking difference between growth on medium from whole rabbit, an animal that is susceptible to the pneumococcus infection, and that on medium from guinea pig, an animal not so susceptible to pneumococcus, is somewhat surprising. For guinea pig medium apparently furnishes substances or conditions suitable for the maintenance of the virulence of the pneumococcus. It is interesting to note, however, that whereas the tissues of one species of animal, or any one tissue of that species prove

TABLE VI, *b*  
*Whole Guinea Pig Medium*  
4 hour interval

No. of transfers	No. of organisms per cc.	No. of organisms constituting fatal dose
84	90,000,000	90
102	60,000,000	6
144	50,000,000	50
84	60,000,000	6
102	80,000,000	8
144	240,000,000	24

satisfactory in maintaining the pathogenicity of a microorganism, the tissues of another species should produce opposite results. If these results were not due to unrecognized variables and hence to an actual difference, further investigation will be needed to tell whether or not this difference is bound up with the broader aspects of varying susceptibility of animal species.

#### *Lung Medium for the Restoration of Virulence*

In a previous study it has been shown that in an avirulent culture obtained from a single cell, grown on milk medium with 2 hours as the interval of transfer, the virulence was increased to a maximum state. In an endeavor to find out whether or not any one organ extract was superior to another for the enhancement of virulence in

*vitro*, media made from those organ extracts which have been shown to furnish conditions suitable for maintenance of virulence were inoculated with avirulent organisms. Briefly, it was found that whole guinea pig medium, calf heart medium, and horse skeletal muscle infusion all failed to enhance the virulence of a relatively avirulent pneumococcus. With lung medium, however, avirulent pneumococci were restored to maximum virulence.

TABLE VII  
*Influence of Lung Medium on Avirulent Culture*

Culture No.	Interval	No. of transfers	No. of organisms per cc.	No. of organisms constituting fatal dose
	<i>hrs.</i>			
5	2	168	110,000,000	110,000
		372	80,000,000	80
		444	30,000,000	30
		600	20,000,000	2
5	4	84	70,000,000	7,000
		180	70,000,000	7,000
		342	130,000,000	13
		378	250,000,000	2
5	8	42	160,000,000	1,600
		108	140,000,000	1,400
		150	180,000,000	180
		189	80,000,000	8
6	4	84	90,000,000	90,000
		216	3,500,000	35,000
		342	200,000,000	2,000
		378	180,000,000	18
		414	350,000,000	35

Two of the avirulent strains studied are included in this report. Culture 5 in Table VII was attenuated to such degree that 1 cc. of the 8 hour culture was necessary to produce a fatal infection in white mice. From the table it is seen that, with 2 hour intervals of transfer on lung medium, after 372 transfers 80 organisms caused the death of a mouse, and after 600 transfers the pneumococci were of maximum virulence. With a 4 hour period of transfer, the same effect was obtained after 378

transfers, and with 8 hour intervals after 189 transfers. Culture 6 was a strain of *Pneumococcus* Type I obtained from a normal throat, and was of such low virulence that 1 cc. failed to kill a mouse but 2 cc. caused a fatal infection. Here again the culture after 378 transfers was raised to maximum virulence.

So far this lung medium has been used on seven different avirulent strains of pneumococcus, including one Type II and one Type III, and in each instance, as judged by ability to kill white mice, there was an increase in the virulence of the organism. No attempts have been made to increase the virulence on lung medium of an avirulent strain developed from a single cell. Hence, it is impossible at present to state whether this process is simply a matter of selection of virulent organisms or whether it is an inherent change in the cell itself. However, it should be borne in mind that the avirulent organisms multiply in this medium seemingly at the same rate as the virulent ones, and thus that the process would appear to be one not of killing the avirulent organisms, but of changing them in such manner that they are capable of producing disease in the experimental animal.

#### DISCUSSION

The work represented in this study is preliminary, and any final deductions in regard to specific tissue activity and virulence are not warranted. However, the results obtained on lung media in relationship to the predilection of the pneumococcus, and the localization of this organism in human pneumonia, are suggestive of a relationship between specific substance and virulence. This relationship gains in likelihood from the fact that, whereas other tissue extracts which have been tried permit at most only the maintenance of virulence, lung extract alone of the various extracts tested supplies conditions favorable to an increase of this characteristic.

That the conditions furnished by the automatic transfer device play a definite rôle in obtaining the results here represented, is clearly shown by a comparison of the results and those obtained by the growth of pneumococci on the various tissue extracts with ordinary transfer methods. Thus during our work in the last 5 or 6 years, media made from different organs have been used to maintain the virulence of pneumococci for the mouse protection test. It was readily found that,

using the customary method of transferring, heart muscle, skeletal muscle of the calf or horse, or lung media alike were all unsatisfactory in maintaining virulence. More than that, when the extracts from different organs, with and without peptone, were used to grow the pneumococcus from repeated mouse passages, no one extract was noticeably superior to the others for maintenance of virulence. Lung medium especially has been tried with just as indefinite results as any other tissue. It would thus appear that conditions which allow a rapid multiplication of the organism in the presence of young viable cells without repression in the so called lag period, really produce significant differences in the maintenance and increase of virulence outside of the animal body.

It should also be pointed out that during this study agglutinability of pneumococci against immune sera was tested almost as a routine procedure. In support of the theory of microbic dissociation, it was found that when the organism decreased in virulence, it became agglutinable in a higher dilution with a given immune serum than in the original virulent state. Conversely, as an avirulent organism became virulent, as in the case of the experiment with lung medium, agglutinability decreased until it reached a constant, that is, a constant dilution of serum with which agglutination took place.

#### CONCLUSIONS

From the study of different tissue extracts as media for the growth of pneumococci used in an automatic transfer device, certain inferences are warranted:

1. Media made from calf lung or heart, or from horse skeletal muscle maintain virulence over a long period of time. Conversely, media made from calf spleen lead to a decrease in virulence.

2. Lung medium causes an increase in virulence of seven strains of pneumococci.

3. Virulence is maintained in normal horse serum; but, it rapidly decreases in immune serum, or in pneumococcus antibody solution, a finding which confirms the work of Stryker. Immune serum freed from protective antibody gives results similar to normal serum.

4. Rabbit medium made from the entire animal apparently is less suitable for the maintenance of virulence of pneumococci than medium made in the same way from guinea pig.

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# V. THE EFFECT OF OXYGEN, NITROGEN, AND CARBON DIOXIDE ON THE VIRULENCE OF GROWING PNEUMOCOCCI\*

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Although the effect of aerobic and anaerobic conditions on the growth of micro-organisms has often been studied, reports on the influence on virulence and pathogenicity are not numerous. Among the earlier workers, Wosnessenski (1), in 1884, showed that anthrax, grown under a pressure of 13 atmospheres of air, slightly increased in virulence for the guinea pig, while a pressure of 15 atmospheres caused a decrease in both growth and virulence. Fränkel (2), in 1888, in a comprehensive study of the effect on the growth of many organisms of bubbling carbon dioxide through the cultures, reported that *tetragenus*, fowl cholera, mouse septi-cemia, and anthrax did not decrease in virulence in a 6 day period. In 1890, Roux and Yersin (3) found that virulence of aerated diphtheria culture showed no decrease as long as incubation was carried out at 35°C., but that incubation at 39.5° was followed by decrease in 2 weeks. In a study of cholera vibrio, Haffkine (4), in 1892, stated that growth at 39° in constantly aerated atmosphere caused attenuation after 2 or 3 days. Lubinski (5), in 1894, found a decrease in virulence for rabbits of *Staphylococcus aureus* grown in atmospheres of pure oxygen, but an increase under anaerobic conditions. Webster (6), in 1925, stated in a dissociation study that, whereas under aerobic conditions the virulent D form of *Past. lepi-septica* with massive inoculation changed to the avirulent G form, under anaerobic conditions this dissociation was greatly inhibited. Soule (7) on the other hand reported, in 1928, that dissociation of *B. subtilis* from S to R form occurred rapidly in from 40 to 60 per cent oxygen, but was inhibited in broth and on agar in the presence of 80 to 100 per cent oxygen. He also showed that dissociation occurred very rapidly under pure nitrogen, but that under nitrogen with 6 per cent carbon dioxide no dissociation at all occurred. Wilson (8), in 1930, showed clearly that *B. aertrycke* under aerobic conditions and partial pressures of oxygen varying from 1 to 21 per cent decreased in virulence rapidly, but that under oxygen pressures from 40 to 100 per cent there was a slight increase in virulence with a maximum

\* This is one of a series of studies carried out in part under a grant by the Influenza Commission of the Metropolitan Life Insurance Company.

in the culture incubated under pure oxygen. Thus from this very short summary of investigations on the influence of aerobic or anaerobic conditions on the virulence of microorganisms, it would seem that conditions for the maintenance of this characteristic vary with different microorganisms.

The present study is a report of investigations on the effect of oxygen, nitrogen, and carbon dioxide on the virulence of pneumococci. Inasmuch as growth in an automatic transfer device was found to be more nearly like that *in vivo* than cultivation by ordinary transfer methods, such an apparatus was used in this study.

The automatic apparatus described in an earlier publication (9) was altered only in that the air inlet on the growth receptacle was connected to a gas supply and the usual cotton plug in this receptacle was replaced by a rubber stopper (Chart 1). The gas was furnished by means of an ordinary laboratory gas bottle arrangement, consisting of a bottle filled with water by means of a siphon. The air inlet to this water bottle was made air-tight with a rubber connection which was in turn clamped by a magnetic release operated by time-clock, which simultaneously opened the clamps on both the water bottle and the medium inlet. By release of the clamps, sufficient water was siphoned into the gas bottle so that between 20 and 25 cc. of gas were forced through the fresh supply of medium in the growth receptacle. It was found that the lag in the gas pressure equilibrium, after water flowed into the gas bottle, was sufficient to allow gas to bubble through this fresh quantity of medium after the growth receptacle was filled. The actual pressure of gas was not measured, but a sufficient quantity was bubbled through the growth receptacle to warrant the assumption that the medium was practically saturated with gas at each interval of transfer. The outlet of the receptacle was sealed by the fluid medium owing to the slightly positive residual pressure in the gas bottle. Consequently the gas pressure remained practically constant throughout the period between transfers.

As in previous work, virulence tests were made by injecting mice with dilutions of culture in logarithmic series. The rate of multiplication was judged by the number of organisms found on rabbit blood agar plates of three different dilutions from each culture.

### *Oxygen, Nitrogen, and Carbon Dioxide*

This experiment was planned to determine the effect of 100 per cent concentrations of oxygen, carbon dioxide, and nitrogen respectively on the virulence of Type I pneumococci. Horse skeletal muscle infusion was chosen as medium for growth, because, as shown in the previous paper (10), it had been found to maintain virulence under aerobic conditions in the automatic transfer device at least over a

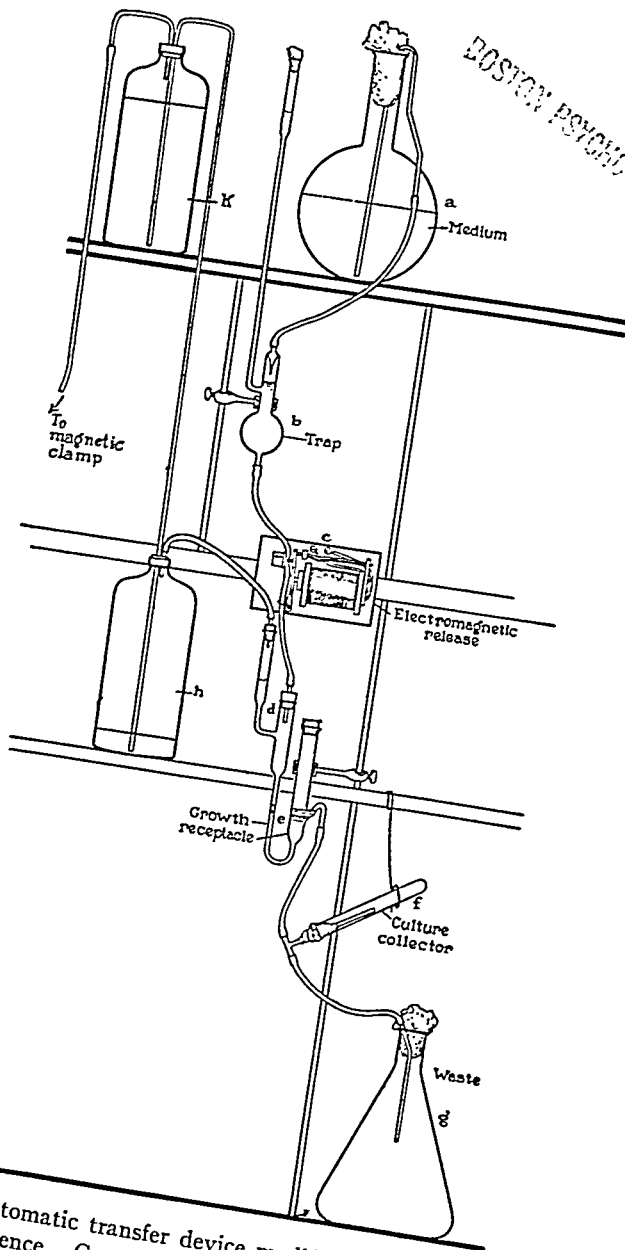


CHART 1. The automatic transfer device modified to study the influence of various gases on virulence. Gases were supplied by means of a gas-water bottle arrangement, H and K. Bottle H, filled with the desired gas, is connected to the growth receptacle and to bottle K which is filled with water. The water bottle is made air-tight with one tubing to the gas bottle and another open to the air through a magnetic clamp similar to C. In operation the two clamps are released simultaneously, and air enters water bottle K as water is siphoned into the gas bottle H, the gas thus being forced through the growth receptacle, bubbling through the fresh supply of medium.

period of 258 transfers. Each apparatus used was regulated to replenish medium and gas every 4 hours. The virulence was tested three times, namely after 72, 120, and 162 transfers. A summary of the results, Table I, shows that the number of organisms averaged highest in the culture under nitrogen, and least under oxygen. Both oxygen and carbon dioxide were found after 162 transfers to reduce the pathogenicity of the organism to such degree that in the former 400,000 organisms were required to kill a mouse, and in the latter, 140,000,000 were not enough to kill. Nitrogen, however, in the same period

TABLE I

*Comparison of Oxygen, Nitrogen, and Carbon Dioxide*

*Horse Skeletal Muscle Medium*

4 hour interval of transfer

Gas	No. of transfers	No. of organisms per cc.	Minimal lethal dilution	No. of organisms constituting fatal dose
Oxygen	72	10,000,000	1-1,000,000	10
	120	8,000,000	1-100	80,000
	162	40,000,000	1-100	400,000
Nitrogen	72	80,000,000	1-10,000,000	8
	120	100,000,000	1-10,000,000	10
	162	90,000,000	1-1,000,000	90
Carbon dioxide	72	16,000,000	1-10,000,000	2
	120	18,000,000	1-1,000	18,000
	162	140,000,000	1 cc. failed	140,000,000

allowed development of the organism to such degree that only 90 organisms proved fatal. The virulence is thus seen to have remained maximum under nitrogen, decreased gradually under oxygen, and dropped precipitately under carbon dioxide.

In addition to the observations of the effect on virulence, dissociation from S to R forms was very clearly shown. At no time in the culture under nitrogen were there found any R forms; but with culture under carbon dioxide, especially toward the end of the experiment, there were very few S forms remaining; and with oxygen the dissociation from day to day showed a progressive increase in the number of

R over S forms. Transplants from S and R forms showed the latter to be granular and for the most part insoluble in bile. However, some of these R forms were found to be virulent for mice. In an experiment in which three R and three S colonies were picked, plated, and then picked again, whereas one of the R colonies was found to be highly virulent and two avirulent, one of the S colonies was of low virulence (1-10,000) and two were of maximum virulence.

It would seem that under the conditions of this experiment, while growth of Type I pneumococci under pure oxygen or pure carbon dioxide causes a decrease in virulence on a medium which normally maintains this characteristic, growth under nitrogen results in no appreciable change in virulence.

TABLE II  
*Variation in Concentration of Oxygen*  
*Horse Meat Infusion*  
4 hour interval of transfer

Gas mixture	No. of transfers	No. of organisms per cc.	Minimal lethal dilution	No. of organisms constituting fatal dose
Oxygen = 5 per cent Nitrogen = 95 per cent	72	10,000,000	1-10,000,000	1
	120	18,000,000	1-1,000	18,000
	162	26,000,000	1-10	2,600,000
Oxygen = 10 per cent Nitrogen = 90 per cent	72	30,000,000	1-1,000,000	30
	120	44,000,000	1-10,000	4,400
	162	16,000,000	1-100	160,000
Oxygen = 20 per cent Nitrogen = 80 per cent	72	20,000,000	1-10,000,000	2
	120	40,000,000	1-100,000	400
	162	20,000,000	1-100,000	200
Nitrogen = 100 per cent	72	80,000,000	1-10,000,000	8
	120	100,000,000	1-10,000,000	10
	162	90,000,000	1-1,000,000	90

*Variation in Oxygen-Nitrogen Mixtures*

In attempting to ascertain the oxygen requirements for maintaining virulence, considerable difficulty in obtaining consistent results was encountered. As stated above, the virulence of this organism was

maintained on horse skeletal muscle medium under aerobic conditions or under nitrogen. So it seemed likely that the optimum concentration of oxygen would be approximately that found in the atmosphere, but that perhaps a smaller pressure of oxygen would not be destructive of virulence. However, in several experiments it was found that a small amount of oxygen in the presence of nitrogen caused a decrease in virulence. One experiment is given as an example. The organism was grown on horse meat infusion under mixtures of respectively, 5 parts of oxygen with 95 parts nitrogen by volume, 10 with 90, 20 with 80, and finally 100 per cent nitrogen. As seen in Table II, under pure nitrogen the virulence remained at a maximum, and, with the 20 to 80 oxygen-nitrogen mixture, was still high, but showed some decrease; with the other gas mixtures, however, there was a very pronounced decrease in virulence. No higher concentration of oxygen in nitrogen has been tried than the 20 to 80 mixture, but it would appear from this experiment, and others, that oxygen in higher concentrations than 20 to 80 might be destructive of virulence in direct ratio to the oxygen concentration. This result with pneumococci is directly opposite to that obtained by Wilson with *B. aertrycke*. For Wilson showed clearly that not only was the virulence of this organism not decreased on pure oxygen, but that in reality it was increased.

#### *Growth with Nitrogen on Various Unfavorable Media under Aerobic Conditions*

This experiment is a study of the influence on virulence of the pneumococcus grown under nitrogen upon media which caused a decrease in virulence under aerobic conditions. Certain media under aerobic conditions, namely spleen, beef infusion, aminoid peptone, and powdered milk, have been found to support growth but cause a decrease in virulence. Because nitrogen caused no decrease in virulence on a favorable medium, these media were tested under nitrogen in the present experiment. Horse meat infusion under nitrogen was used as a control medium. As seen in Table III, the spleen medium, tested after 42, 78, and 120 transfers with 4 hour intervals of transfer, showed a decrease in virulence of the organism, but not so rapid as was reported in the previous aerobic study. With beef infusion, the results are more satisfactory; for maximum virulence

was maintained throughout 150 transfers, and even after 228 transfers the decrease was only approximately 100-fold. Results similar to these were obtained with aminoid peptone: although there was a definite decrease, the rate of decrease was not so rapid as in other experiments with this medium under aerobic conditions.

TABLE III  
*Growth in the Presence of Nitrogen on Certain Media Which Failed to Maintain Virulence under Atmospheric Conditions*  
4 hour interval of transfer

Media	No. of transfers	No. of organisms per cc.	No. of organisms constituting fatal dose
Spleen	42	24,000,000	24,000
	78	12,000,000	12,000
	120	1,000,000	100,000
Beef infusion	60	10,000,000	10
	102	20,000,000	2
	150	24,000,000	24
	198	50,000,000	50,000
	228	10,000,000	1,000
Aminoid peptone	48	200,000,000	200
	102	8,000,000	800
	144	3,000,000	3,000
	48	400,000,000	40
Powdered milk	102	60,000,000	6
	144	80,000,000	800
	186	70,000,000	700
	102	80,000,000	8
Horse meat infusion (control)	120	100,000,000	10
	162	90,000,000	90
	204	85,000,000	85

It has been shown that under aerobic conditions fresh milk medium supplies conditions or substances requisite for maintenance and even increase of the virulence of certain strains of pneumococci. On the other hand, a solution of commercial powdered milk failed to maintain virulence under aerobic conditions, no doubt due to alteration during



the process of evaporation similar to the effect of heat on whole fresh milk. For in our second paper on virulence (11), it was found that milk which was heated for various lengths of time became unsuitable for maintaining virulence, in direct ratio to the length of time heated; that is, the longer the period of heating, the more rapid the decrease in virulence. However, when powdered milk was used as medium for growth of pneumococci under nitrogen, virulence was maintained at maximum for at least 120 transfers, with apparently only a 100-fold decrease after 186 transfers.

It is thus seen that, although media unsuitable for maintenance of virulence under aerobic conditions became more favorable under nitrogen, at least the degree of anaerobiosis supplied under the conditions of this experiment did not suffice for maintenance of maximum virulence. Furthermore, other experiments have shown that avirulent organisms grown on these media with nitrogen failed to increase in virulence.

#### *Growth with Nitrogen on Guinea Pig, Rabbit, and Immune Mice Media*

It was reported in the previous paper that media made from whole guinea pig and from whole rabbit respectively acted differently in their influence on virulence of pneumococci grown under aerobic conditions; that is, while guinea pig medium maintained virulence, rabbit medium failed in this respect. To test the effect of anaerobic conditions, media made from these animals, as well as from discarded mice which had survived pneumococcus infection, and also from rabbit muscle, were used to grow virulent pneumococci under a pressure of 100 per cent nitrogen. Just as was found under aerobic conditions, guinea pig medium (Table IV) maintained the virulence to a higher degree than did rabbit. However, it is apparent that guinea pig medium is not as satisfactory with nitrogen as it was found to be in the presence of atmospheric oxygen. Rabbit muscle, found aerobically unsuited for maintenance of virulence, is here shown to yield the same result under nitrogen. The discarded mice medium showed first a decrease in virulence similar to that with growth on immune serum; but unlike the effect of immune serum, the organisms on this medium later increased in virulence so that after a period of 246 transfers a very high, though not maximum, degree of virulence was attained.

Here again it is found that media which under aerobic conditions are unsuitable for maintenance of virulence fail to improve under nitrogen. The one exception is perhaps the experiment on discarded

TABLE IV  
*Growth in the Presence of Nitrogen on Guinea Pig, Mouse, and Rabbit Media*

Media	Transfer interval hrs.	No. of transfers	No. of organisms per cc.	No. of organisms constituting fatal dose
Guinea pig	4	42 90 120 186 246	80,000,000 2,000,000 300,000,000 60,000,000 60,000,000	80,000 20 300 60,000 60,000
Discard mice	4	42 90 120 186 246	120,000,000 12,000,000 2,000,000 4,000,000 120,000,000	12,000 1,200 200 400 1,200
Rabbit (including skin)	2	36 84 180 240	400,000,000 60,000,000 12,000,000 70,000,000	40 600 120,000 70,000,000
Rabbit (without skin)	2	36 84 180 240	330,000,000 200,000,000 5,000,000 60,000,000	33 200 5,000 600,000
Rabbit muscle	4	36 78 126 174 204	40,000,000 30,000,000 20,000,000 22,000,000 80,000,000	40 300,000 200,000 22,000,000 80,000,000

mice as medium, in which there was first a decrease and then an increase in virulence. This exception suggests a possible gradual immunizing effect in the microorganism against the antibodies present in mice actively immune against pneumococci.

*Effect of Temperature*

During this study the mechanism controlling the temperature of the incubator room became defective with the result that the temperature increased over a period of 2 days to 41°C. This change in temperature was not noted until after mice had been injected with the cultures from the transfer machines that were operating at that time. It was observed that all cultures irrespective of the media had decreased greatly in virulence during the period of higher temperatures. Such destructive influence of temperature on virulence has been noted by many investigators. Consequently it is our purpose here simply

TABLE V  
*Effect of Temperature (Aerobic Conditions)*  
4 hour interval

Date	Temperature	No. of transfers	No. of organisms per cc.	Minimal lethal dilution
	°C.			
Nov. 3.....	36.5	6	70,000,000	1-10,000,000
Nov. 5.....	37	18	20,000,000	1-10,000,000
Nov. 6.....	37.5	24	34,000,000	1-10,000,000
Nov. 7.....	38	30	12,000,000	1-10,000,000
Nov. 8.....	38.75	36	10,000,000	1-1,000,000
Nov. 9.....	39.2	42	18,000,000	1-1,000,000
Nov. 10.....	39.5	48	30,000,000	1-1,000
Nov. 11.....	41	54	20,000,000	1-1,000
Nov. 12.....	42	60	12,000,000	1 cc.
Nov. 13.....	42	66	8,000,000	1 cc. failed

to present an experiment to show the effect of a gradual increase in temperature on the virulence of pneumococci when transferred at short intervals on medium which under aerobic conditions maintains maximum virulence at 37.5°C.

Inasmuch as horse meat infusion was found to maintain virulence through a relatively long period, this medium was used for the experiment. By four adjustments during each day of a 10 day period, the temperature was increased gradually from 36.5°C. on the 1st day to 42°C. on the 10th day. Temperatures were read in the morning of each day at the time test samples were removed. It was found, Table

V, that although the number of organisms varied somewhat from day to day, there was a definite decrease only after the 1st day at 42°. Yet at this high temperature there were as many as 12,000,000 organisms per cubic centimeter. Virulence remained maximum up to 38°. From that time on, however, there was a rapid decline so that at a temperature of 42°, 1 cc. of culture, containing 12,000,000 organisms, was necessary to cause a fatal infection in a mouse. Subsequently this avirulent organism was passed repeatedly through mice in the usual manner without any perceptible return to its former virulent state. It would thus seem that the change which had taken place was apparently an example of a complete dissociation from S to R form.

#### CONCLUSIONS

The growth of a virulent Type I pneumococcus in an automatic transfer device on a medium which aerobically maintains virulence results in a decrease in the presence of pure oxygen or pure carbon dioxide, but in no change in the presence of nitrogen. Whereas partial pressures of oxygen, that is 5 parts with 95 parts nitrogen by volume, and 10 parts with 90 parts nitrogen, resulted in a decrease in virulence, with 20 parts oxygen and 80 parts nitrogen, the decrease was almost negligible. Media which under aerobic conditions failed to furnish suitable constituents for maintenance of virulence, that is spleen, aminoid peptone, powdered milk, beef infusion, whole rabbit, and rabbit muscle, also failed under nitrogen. Media from whole guinea pig kept the organism virulent for 246 transfers under nitrogen, but with some decrease. Media made from discard mice, survivors of pneumococcus infection, furnished conditions under nitrogen, which caused first a decrease of virulence, but later after 246 transfers, a restoration to a more virulent state. Gradual increase in temperature over a period of 10 days from 36.5°C. to 42°C. in a sample of medium, which was otherwise suitable for maintenance of virulence under aerobic conditions, resulted in a decrease in virulence.

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# CELLULAR REACTIONS IN THE MENINGES OF RABBITS TO TUBERCULO-LIPOID, PROTEIN, AND POLYSACCHA- RIDE, COMPARED WITH THE EFFECTS OF TUBERCLE BACILLI

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PLATE 1

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The experiments presented in this paper involve the study of meningitis produced in rabbits by the introduction of living and killed tubercle bacilli into the subarachnoid space, as compared with the effects of the injection of various extracts from the tubercle bacillus. Every extract tested has produced some symptomatic or pathological change. The tests have been made in tuberculous and in normal rabbits. The differences in their reactions have proved to be only quantitative.

Recent experimental studies of tuberculous meningitis have been made by Opie (1), Manwaring (2), Austrian (3), Foot (4), Kasahara (5), and Soper and Dworski (6, 7). In 1925, Soper and Dworski (6) injected tubercle bacilli into the meninges of tuberculous rabbits. They obtained striking results, showing that the phenomena attending superinfection manifest themselves in the meninges of the rabbit as well as in the other serous membranes and organs of various experimental animals. They found that with high superinfecting doses death was probably hastened by an allergic state; with medium doses the period of survival was double that of the controls; while with relatively small doses the superinfected animals often showed high resistance and survived a long period in comparison with the controls, which had received a single inoculation in the meninges. Examination of the spinal fluid indicated that in the superinfected animals an intense meningeal reaction occurred immediately, with increased red and white cell counts which persisted for a few days after inoculation but gradually diminished. In the controls the counts were relatively low at first and then followed an irregular course upward as the disease progressed. In 1930 they published further work (7) showing that by using very small doses for superinfection, the rabbits were able to survive the disease, while all the control animals which had received a single inoculation into the meninges died.

In 1929, Rich and McCordock (8) in their studies on the relation of allergy to immunity and to the lesions in tuberculosis gave evidence to show that, clinically, meningitis occurs when a tuberculous focus in the brain or meninges ruptures, liberating living bacilli into the subarachnoid space. They think that the bacilli do not pass directly from the blood stream to the spinal fluid, but that the involvement of the meninges is due to the fact that some focus which had developed in a blood vessel or in the periphery of the brain becomes caseous and ruptures into the subarachnoid space, thereby liberating organisms into it. This idea fits well with the observations made in experimental work, for intravenous inoculations with living organisms rarely result in meningitis, and, when it occurs, a caseous area can be located which has ruptured into the subarachnoid space.

The first of the present studies was made with living tubercle bacilli.

### *Methods*

The organisms were introduced by the postorbital route, but this was soon given up since it was too difficult to obtain cerebrospinal fluid and to determine the exact position of the point of the needle. It is essential to withdraw as much fluid as is injected in order to avoid the phenomena due to increased intracranial pressure. Cisternal puncture through the atlantooccipital ligament was found feasible. Later it was discovered that this method had been described in 1919 by Wegeforth, Ayer, and Essick (9). Better results are obtained if, after entering the skin and muscles over the area, the stylet of the needle is withdrawn before piercing the ligament. This affords the advantage of obtaining a free flow of fluid as soon as the point of the needle enters the cisterna cerebellomedullaris, with avoidance of the possibility of entering the medulla before one is aware that the needle has passed through the ligament. After one has become familiar with the method there is no need for an anesthetic, since it causes little disturbance of the animal.

The bovine tubercle bacillus used was the Strain B-1, originally isolated by Dr. Theobald Smith and obtained from the Saranac Laboratory. It was known to be virulent for rabbits. The suspension was prepared from a weighed quantity of bacilli grown on Petroff's egg-gentian violet medium. Normal saline was added to make a suspension, the organisms were then counted in the Petroff-Hausser bacterial counting chamber, and a further dilution made until 0.2 cc. contained the desired number (usually 500,000). If any large clumps were present, the suspension was filtered and recounted.

Heat-killed organisms were prepared from a subculture of the B-1 strain as above. 100 mg. (moist weight) of organisms were ground in 10 cc. of freshly prepared normal saline. The suspension was heated in a water bath at 70°C. for 1 hour. Dilutions were made so that 0.2 cc. of the final suspension contained the desired number of organisms.

Formalin-killed organisms were also prepared from a subculture of B-1 described above. 400 mg. (moist weight) of organisms were suspended in 20 cc. of normal

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saline. Sufficient formalin was added to make a concentration of 0.4. This was incubated at 37°C. for 10 days. The suspension was then centrifuged and the fluid decanted. The remaining formalin was removed by washing times with freshly prepared normal saline solution, and the organisms were suspended in normal saline solution with 0.5 per cent phenol added as a preservative. Dilutions of this suspension were made so that the amount used for inoculation contained the desired number of organisms.

The lipoid used for the subarachnoid injections was the phosphatide A-3, isolated from the bovine tubercle bacillus by Dr. R. J. Anderson (10) at the Sterility Chemistry Laboratory of Yale University. This extract has proved to have the specific biological property of producing tubercular tissue, that is, epithelioid cells, when introduced subcutaneously and intraperitoneally in rabbits and guinea pigs (11).

The tuberculo-protein was received from the H. K. Mulford Biological Laboratories and was designated as MA-100. This was prepared from the filtrate of cultures of the human tubercle bacillus, Strain H-37.

Fresh tissue from the dura, pia, and brain was examined immediately after removal at autopsy, using supravital staining with neutral red and Janus green (12). The entire brain and upper portion of the cord were fixed in 10 per cent formalin for 2 days. After this, they were cut into blocks, washed, dehydrated, and embedded in paraffin. The sections were stained with hematoxylin and eosin. The other organs were fixed in Helly's mixture (Zenker base with 5 per cent formalin), and treated as above. Needless to say, all the animals were kept under the same conditions as to air, sunlight, feeding, and general care.

The occurrence of spontaneous encephalitis in apparently normal rabbits is well known. It was first described and related to a protozoan parasite by Wright and Craighead (13) in 1922 and has since been repeatedly studied (14-19). The lesions consist of round cell infiltrations of the meninges, perivascular infiltrations in the brain tissue, necrotic areas and cysts in which the parasite may be seen. They may closely resemble tubercles with caseous centers and were frequently encountered in this study.

*Tuberculous Meningitis in Rabbits*

Fifteen normal, healthy, adult rabbits were inoculated intrameningeally with 500,000 (0.0000125 gm.) undissociated tubercle bacilli, Strain B-1, from a 3 weeks' growth of Subculture 223. All the animals appeared well after operation. There were no symptoms of meningitis or of paralysis. At short intervals after inoculation they were killed to determine the cellular changes.

The cellular reactions, both to tubercle bacilli themselves and to the phosphatide extracted from them, were characterized by a type of cell which merits especial description.



The cell was predominant in the reaction in the pia in the early stages of the disease, and later was found in the cerebrospinal fluid. It is shown in a photograph of living cells from the pia in Fig. 1. Its size, as can be judged from the red corpuscle in the lower right hand corner of the figure, was about that of an actively phagocytic clasmatocyte or a large epithelioid cell. The cytoplasm was so completely filled with vacuoles that the nucleus was entirely obscured. The vacuoles were of even size, uniformly stained, and reacted with the neutral tint of vital neutral red. The reason for the variations in the photograph is that the content of the vacuoles was highly refractive, suggesting lipoidal material, and, as is well known, highly refractive globules appear black when the focus is not in the equatorial plane.

It is difficult to classify this cell. It differs from the actively phagocytic clasmatocyte usually found in the tissues, the latter having vacuoles of different size which show the complete range of color of the dye at different pH values, namely, from red to yellow. Likewise, it has none of the characteristics of the typical monocyte.

Until the 15th day after the intrameningeal injection of the living bovine tubercle bacilli, the animals showed no abnormal symptoms. Those allowed to live 15 days or longer had first a weakness of the hind quarters, became progressively worse, and finally developed partial paralysis by the 27th day.

Although the rabbits showed no symptoms of meningitis until some time after injection, there was a definite cellular response which occurred during this incubation period.

On the 1st day after the inoculation, supravital studies of the pia revealed the characteristic cell described above as having the vacuoles of even size and uniform staining reaction with the vital neutral red. There was an abundance of these cells throughout the pia and some in the dura. On the 2nd day the specimens could not be distinguished from those of the preceding day. By the 3rd day a definite increase in the number of lymphocytes and plasma cells in the specimen from the pia was observed. At the 4th day a few of the characteristic vacuolated cells just described showed certain changes; namely, an increase in the number of the vacuoles, with a corresponding decrease in their size. The vacuoles at this time were larger than those of the cell designated by Sabin *et al.* (11) as the coarse vacuole epithelioid cell. The predominance of lymphocytes and plasma cells was observed. The next day (the 5th), there did not seem to be any increase in the number of cells with the smaller vacuoles. The cells with larger vacuoles were present in far greater proportion than those with the smaller. A scraping from the medulla and midbrain contained sheets of monocytes, some of which very nearly approached typical epithelioid cells (Fig. 2). Otherwise there was no change in the findings. For the period from the 6th to the 15th day, it was difficult

to distinguish any daily change in the cellular picture other than a steady increase in the number of lymphocytes and plasma cells in the specimens from the pia. By the 15th day, however, a great number of the mononuclear cells containing vacuoles of even size and uniform staining reaction showed an increase in number and a decrease in size of the vacuoles, which was a reversal of the previous findings. At the end of 27 days, only an occasional cell with the large vacuoles was observed. The vacuoles in most of the cells of this type had diminished in size until they were the size found in the coarse vacuole epithelioid cell. These cells correspond to those seen in Fig. 4.

Histological sections from these animals showed the visceral organs to be normal during the entire course of the experiments. The sections from the brain and cord during the first 13 days revealed a progressive lymphocytic infiltration of the meninges extending into the sulci. By the 13th day the vessels around the periphery of the cord for a short depth showed marked perivascular lymphocytic infiltration. On the 19th day the infiltration had greatly increased and there was beginning tubercle formation in some areas of the meninges. Sections on the 27th day showed extensive tuberculous processes in the meninges and invading the brain substance.

Ten rabbits were inoculated intrathecally with 800 organisms. Some of these animals are still living so that the data are incomplete but it is clear that both the period before the onset of symptoms, and the survival, are longer than in animals inoculated with the greater number of organisms.

#### *Subarachnoid Inoculation of Heat-Killed and Formalin-Killed Tubercle Bacilli*

Varying doses of killed organisms were injected in a small series of rabbits to determine whether there was any distinguishable difference in the reactions to bacilli killed by heat, and to those killed with formalin. In Table I are presented the findings in the animals inoculated intrathecally with heat-killed tubercle bacilli.

In the animals receiving the heat-killed organisms, the immediate reaction was similar to that with the living organisms. The predominant cell in the pia, as seen in fresh preparations, was the cell with even sized vacuoles taking the uniform stain with the vital neutral red. The vacuoles were of the coarse variety. This preparation could not be distinguished from that obtained in the first 6 days after

the injection of living bacilli. Acid-fast stains of the pia in one animal 2 days after the last injection demonstrated acid-fast debris, but no whole organisms. Phagocytosis of this debris could not be detected.

TABLE I

*Results of Intrameningeal Injections of Heat-Killed Tubercle Bacilli into Normal Rabbits*

No. of rabbit	Amount of bacilli	Interval between injection and autopsy	Course	Autopsy findings
R 1787	8 million	1 day	Operative trauma. Killed	Brain showed neither edema nor hemorrhage. Viscera normal. Supravital studies of the pia showed great numbers of the meningeal type of early epithelioid cell and a few lymphocytes and plasma cells
R 1786	8 million. 2 days later 8 million	4 days	Operative trauma at 2nd injection. Killed	Brain covered with a blood clot. Viscera normal. Supravital studies of the pia showed great numbers of the meningeal epithelioid cells. Lymphocytes were pronounced in both pia and dura
R 1791	8 million. 2 days later 8 million. 2 days later 8 million	4 days	Killed on day of last injection	Brain slightly injected; no edema and no hemorrhage. Viscera normal. Supravital studies of the pia showed the same cellular reactions as in R 1786
R 1782	8 million	28 days	Animal normal. Killed	Brain appeared normal; no edema and no hemorrhage. Viscera normal. Supravital studies of the pia showed enormous numbers of the meningeal type of early epithelioid cell. No diminution in the size of the vacuoles of these cells
R 1756	1 million	33 days	Animal normal. Killed	Same as in R 1782

TABLE I—*Concluded*

No. of rabbit	Amount of bacilli	Interval between injection and autopsy	Course	Autopsy findings
R 1757	1 million. 5 weeks later 8 million	78 days (41 days after last injection)	Normal until 65th day. Killed after 13 days illness, with weakness and loss of coordination	Brain appeared normal. Viscera normal. Cerebrospinal fluid from the 4th ventricle contained sheets of epithelioid cells and stimulated monocytes. Supravital studies of the pia revealed epithelioid cells with small, uniform, even sized vacuoles. Increase in lymphocytes. Sections revealed tubercles in the meninges and an invasion of the brain with lymphocytes from the pia
R 1783	8 million. 1 month later 8 million	93 days (65 days after last injection)	Normal until 68th day. Killed in <i>extremis</i> after 25 days illness, with weakness and loss of coordination	Same as in R 1757 except a more extensive tuberculous process in the meninges and the beginning of an invasion of the brain with tubercles

At the end of 1 month the animals appeared normal. There was no loss of weight, nor other signs of illness. Fresh preparations of the pia at this time revealed a predominance of the same cells as described above with the equal sized and uniformly staining vacuoles. The vacuoles did not seem to have diminished appreciably in size. The number of lymphocytes and plasma cells was far in excess of that found in earlier studies. Again, it was difficult to distinguish this reaction at the end of a month from that obtained during the middle of the incubation period after inoculation with living bacilli. Histological studies showed that the visceral organs were normal. The sections of the brain showed lymphocytic infiltration of the meninges of a mild character. There was no involvement of the brain substance and no perivascular reaction.

The two remaining rabbits, R 1757 and R 1783,<sup>1</sup> continued in good health until the 65th day after the first inoculation when R 1757 suddenly began to lose weight, became weak in the hind quarters and progressively worse. 13 days after this acute exacerbation, on the 78th day after injection, the animal was killed. R 1783 had a similar history. This animal became ill on the 68th day and, being *in extremis* after progressive weakness for 25 days, was killed on the 93rd day after injection. In these two animals no gross tubercles were observed in the brain or meninges. Scrapings from the ventral surface of the medulla, studied supravitaly, showed numerous stimulated monocytes and typical epithelioid cells. Cerebrospinal fluid removed directly from the fourth ventricle as soon as the cranial cavity was opened showed these same cells. Fig. 3 shows a sheet of these cells in spinal fluid. Preparations of the pia, using the same technique, revealed the coarse vacuole cell described previously. Plasma cells and lymphocytes were present in unusually large numbers. The dura was thickened but contained no abnormal cellular elements. Histologically, the brain condition closely resembled that of the last 2 animals (R 2029, R 2030) of the experiments with living bacilli.

Two rabbits were inoculated with formalin-killed organisms. Neither showed abnormal signs. One was killed at the end of 1 month and exhibited pathological features similar to those of the heat-killed group at this period. The other rabbit was finally killed on the 129th day to terminate the experiment and at autopsy was found to be normal except for an occasional cell with equal sized vacuoles of both large and small type in the pia.

The number of animals in these two groups is too small to warrant conclusions. One can only say that the evidence points to the fact that heat-killed organisms are not innocuous when introduced into the meninges of the rabbit. They produce lesions similar to those obtained with living organisms, but the time required for development is greatly prolonged. Further studies of these reactions are being made.

#### *Reaction in Normal and Tuberculous Rabbits to Subarachnoid Introduction of Bovine Phosphatide A-3*

Sabin and her associates (11) have injected as much as 80 mg. of phosphatide intraperitoneally in rabbits. It was plain that so much could not be injected into the subarachnoid space.

<sup>1</sup> These are serial numbers of the work of the department covering a term of years.

A dose of 7.5 mg. of the phosphatide A-3 suspended in 1.5 cc. of freshly distilled water was introduced into one animal (R 1591). The rabbit died in 21 hours with signs of increased intracranial pressure. The autopsy findings in the brain were chiefly those of a non-specific reaction to a foreign body. It was found that the optimum amount of fluid for each subarachnoid injection was 0.2 cc. Since the phosphatide was sparingly soluble in water, the maximum dose was limited to 2.0 mg. in 0.2 cc. of fluid.

Nine normal (Table II) and seven tuberculous rabbits (Table III), were given phosphatide A-3 into the subarachnoid space.

The total amount introduced in each animal varied from 0.1 mg. (R 1605 and R 1606) to 12.0 mg. (R 1882); and the amount of phosphatide in the smallest dose (0.1 mg.) represents that in 1.6 mg. of bacilli, which is 160 times the amount of living bacilli which we used in these experiments. The largest dose represents nearly 20,000 times this amount. It was found necessary to grind the phosphatide in a mortar while adding water drop by drop. In this manner a uniform suspension was obtained, which did not precipitate on standing. Unfortunately in the tuberculous group two of the rabbits (R 1741 and R 1743) were traumatized in later operations as the needle punctured the medulla and caused hemorrhage. These were autopsied immediately afterward, and the fresh blood in the cisterna did not obscure the cellular response in the fresh tissues. None of the animals suffered from secondary infections.

In the studies with the phosphatide there have been no complications from the presence of a diluting menstruum; the sterile water used was itself inert to the connective tissues (11). The amount of reaction from the mechanical effects of the operation and the injection of 0.2 cc. of fluid in the cisterna was controlled by injecting one animal, R 1607, with a weak (non-toxic) solution of neutral red (0.0025 per cent). There was no rise in temperature after this operation and the organs were normal at autopsy. Five rabbits were also given 0.2 cc. of a filtrate obtained by grinding a normal rabbit's brain in normal saline and filtering through double layers of Whatman's No. 5 filter paper. Culture of the filtrate was sterile. In these rabbits there was no rise in temperature and they remained normal.

The phosphatide itself could not be sterilized, but other precautions for sterility were taken, and there were no signs of a contaminating infection after its use. In the animals receiving the phosphatide A-3, the early reaction was characterized by the cells with even sized and uniformly staining vacuoles. These cells were identical with those

TABLE II

*Results of Intrameningeal Injections of Tuberculo-Phosphatide into Normal Rabbits*

No. of rabbit	Amount of phosphatide in distilled water	Interval between injection and autopsy	Course and autopsy findings
R 1591	7.5 mg. in 1.5 cc.	Died in 21 hrs.	No symptoms after injection. Appeared ill following morning; head retracted to left; later clonic generalized convulsions, vertical nystagmus, coma, and death. Viscera normal. Brain edematous and hyperemic. Supravital studies of the pia and dura showed clasmatocytes
R 1605	0.1 mg. in 0.2 cc.	Killed 4th day	No symptoms after injection. Following day seemed lethargic and had hyperpnea. Recovered on the 2nd day and remained normal until killed on the 4th day. Viscera normal. Brain showed no edema and no hyperemia. Supravital studies of the pia revealed a number of meningeal epithelioid cells with coarse vacuoles. There were moderate numbers of lymphocytes
R 1606	0.1 mg. in 0.2 cc.	Killed 10th day	No symptoms after injection. Animal remained well until the 10th day, when it was killed. Viscera normal. Brain hyperemic but no edema. Supravital studies of the pia showed many epithelioid cells, both those with coarse and those with fine vacuoles. Moderate increase in lymphocytes
R 1638	1 mg. in 0.2 cc.	Killed on 24th day	No symptoms after injection. Animal remained well until the 24th day, when it was killed. Viscera normal. Brain normal in the gross. Supravital studies of the pia showed monocytes, epithelioid cells, and many giant cells with fine vacuoles. Increase in number of lymphocytes
R 1723	1 mg. in 0.2 cc.	Killed on 24th day	No symptoms after injection. Animal remained well until the 24th day, when killed. Brain normal in the gross. Supravital studies of the pia showed an increase in the meningeal type of epithelioid cells. Many clasmatocytes and moderate numbers of lymphocytes

TABLE II—*Concluded*

No. of rabbit	Amount of phosphatide in distilled water	Interval between injection and autopsy	Course and autopsy findings
R 1724	2 mg. in 0.2 cc.	Killed on 24th day	Same as R 1723
R 1725	2 mg. in 0.2 cc.	Killed on 24th day	Reactions same as in R 1723 and R 1724
R 1607	5 injections of 1 mg. each in 0.2 cc.	Killed on 32nd day	Animal remained well for 32 days. Killed 32 days after the 1st injection and 7 days after the last. Peritoneal cavity contained 30 cc. of clear fluid free of cells. No evidence of peritonitis. Brain normal in the gross. Supravital studies of the pia same as in preceding three animals

found after the introduction of heat-killed organisms both in the early reaction and after 1 month. Fig. 1 illustrates these cells well, although this particular photograph was from the pia of a rabbit killed 1 month after inoculation with heat-killed tubercle bacilli.

At the end of 1 month (24 to 32 days) the findings in the normal animals injected with phosphatide remained unchanged. The predominant cell was that with the coarse vacuoles. There was no diminution in the size of these vacuoles. This observation indicates that the meningeal cells break down the phosphatide much more slowly than the corresponding cells elsewhere.

In the tuberculous group at 16 days the picture also was unaltered.

One animal (R 1739) was finally killed to terminate the experiment, 99 days after the first injection of phosphatide. At that time the rabbit appeared in excellent health, had gained weight, and showed no signs of infection in spite of the fact that it had had tuberculosis for 4 months. At autopsy the brain appeared normal in the gross. A thick brown exudate covered the medulla and midbrain. A scraping from this contained the typical cells as described before, but the vacuoles were now much smaller in size (Fig. 4). It is interesting to note that some of these cells appeared to contain a lipoid showing myelin-like figures, which has been found to characterize the phosphatide itself (20, 21). The pia contained some of these cells but showed no marked stimulation. The other organs had extensive generalized tuberculosis.



TABLE III

*Results of Intrameningeal Injections of Tuberculo-Phosphatide into Tuberculous Rabbits*

No. of rabbit	Interval between infection with tubercle bacilli and 1st injection of phosphatide	Amount of phosphatide in distilled water	Interval between injection of phosphatide and autopsy	Course and autopsy findings
R 1718	26 days. Skin test positive 16th day	2 mg. in 0.2 cc.	1 day	No symptoms immediately after injection and no rise in temperature. No paralyses or signs the evening after the injection but died during the night. Extensive pulmonary tuberculosis. Postmortem changes in the brain too marked to obtain any studies of the brain but no hemorrhages seen
R 1741	22 days. Skin test positive 18th day	1 mg. in 0.2 cc.	2 days. Killed at a 2nd operation	No symptoms immediately after injection but rise in temperature to 105.9°F. in 4 hrs. No paralyses. Killed 2 days later by trauma at a 2nd operation. Pulmonary tuberculosis. Moderate hyperemia of brain; no edema or old hemorrhages, but fresh clot in ventricles. Supravital studies of the pia showed masses of meningeal epithelioid cells with coarse vacuoles. Few lymphocytes
R 1743	22 days. Skin test positive 18th day	2 injections of 1 mg. each in 0.2 cc.	2 days. Killed by trauma of 2nd operation	No symptoms immediately after injection but temperature rose to 107.1°F. in 5 hrs. No paralyses. At 2nd injection 2 days later the cerebrospinal fluid removed had blood and animal showed loss of coordination and convulsions and died in 1 hour. Pulmonary tuberculosis. Brain showed hemorrhage in cisterna and trauma of the cord. Supravital studies of the pia showed masses of meningeal epithelioid cells with coarse vacuoles

TABLE III—*Continued*

No. of rabbit	Interval between infection with tubercle bacilli and 1st injection of phosphatide	Amount of phosphatide in distilled water	Interval between injection of phosphatide and autopsy	Course and autopsy findings
R 1705	52 days	2 mg. in 0.2 cc.	3 days. Killed in <i>extremis</i>	No symptoms immediately after injection but temperature rose to 107°F. in 5 hrs. Weakness and loss of coordination developed on the 3rd day and the animal was killed by intravenous injection of air. Pulmonary tuberculosis. Brain showed slight hyperemia; otherwise same as in R 1743
R 1737	110 days	2 mg. in 0.2 cc.	5 days. Died	Animal in very poor condition at time of injection. No symptoms immediately after but died on the 5th day. Extensive generalized tuberculosis elsewhere but postmortem changes too great for study of the brain
R 1740	22 days. Skin test positive 18 days	2 injections of 1 mg. each, 2 days apart	16 days. Killed	No symptoms immediately after injection but the temperature rose to 107° and 106.3°F. in 5 hours after the injections. Killed on the 16th day after the 1st and 14 days after the 2nd injection while still in good condition. Pulmonary tuberculosis. Brain slightly hyperemic. Supravital studies of the pia showed great numbers of meningeal epithelioid cells with the coarse vacuoles

TABLE III—*Concluded*

No. of rabbit	Interval between infection with tubercle bacilli and 1st injection of phosphatide	Amount of phosphatide in distilled water	Interval between injection of phosphatide and autopsy	Course and autopsy findings
R 1739	22 days. Skin test positive 18th day	2 injections of 1 mg. each, 2 days apart 1 injection of 2 mg. 16 days after 1st injection, 14 days after 2nd	99 days. Killed	No symptoms immediately after injection but subsequent rise in temperature. Animal remained in good condition and gained 800 gm. in weight. Killed on 99th day after the first and the 83rd day after the last injection. Pulmonary tuberculosis. Brain normal in the gross but a thin, brown exudate over the medulla. Supravital studies of the pia over the midbrain showed epithelioid cells whose vacuoles were much smaller than in any of the previous specimens after the phosphatide. These cells were like the typical coarse vacuole epithelioid cells of other tissues. Pia over the brain showed a few of the same cells

The striking result of the intrameningeal injection of the tuberculo-phosphatide was the production of the cells with vacuoles of even size stained uniformly with vital neutral red. This response was more pronounced than after the introduction of living and heat-killed bacilli. These cells remained unchanged at the end of 1 month, but, finally at the end of 3 months, the vacuoles of the cells had diminished in size until they were the size found in the coarse vacuole epithelioid cell, and corresponded exactly to the type observed in the terminal stages after the introduction of living bacilli. It is interesting to note the relatively great length of time after phosphatide that the cells in the meninges take to break down the large vacuoles into small ones.

Studies of the cells of the peripheral blood of these rabbits were made at various times during the course of the experiment. In the control group there was no

essential change in the percentage or totals of the differential count. There was no tendency for a rise in monocytes. One normal animal, R 1607, receiving phosphatide intrameningeally, had a shower of monocytes for 1 day (22 per cent with a total of 3,058), but with this exception, the range of monocytes in this animal during the period of injections was 29 to 1,090 cells and the average total was only 906 monocytes, as compared with 928 before. The red cells remained normal. In the tuberculous group the blood changes were characteristic of advancing tuberculosis with an irregular rise in monocytes.

Precipitin tests (20) on the spinal fluid were negative in all cases. R 1882, which received a total of 12.0 mg. of phosphatide, had no precipitin reaction of the blood serum. This test was performed in order to find any evidence of precipitins to the phosphatide in the blood serum.

### *The Reaction in Normal and Tuberculous Rabbits to Subarachnoid Injections of the Tuberculo-Protein, MA-100*

A normal rabbit, R 1639, was injected intrathecally with 1.0 mg. of tuberculo-protein MA-100. The animal appeared normal after the operation. The temperature rose from 102.8°F. before injection to a peak of 106.4°F. in 5 hours. The following day the temperature remained around 104.0°F., but on the 2nd day after injection it was below the level previous to the operation. 12 days later, the animal received a second dose of 1.0 mg. of MA-100. The temperature rose from 99.8° before injection to a peak of 105.4° in 3½ hours, but was normal the following day. The rabbit was then given protein every 3rd day. The third injection of 1.0 mg. gave a rise in temperature from 101.8° to 103.2° in 2 hours without a peak and remaining within the limits of normal variation. The fourth injection of the same 1.0 mg. dose on the 3rd day following gave only a variation in temperature within the normal limits (Chart 1). Apparently the animal had become refractory so that it failed to react to this dose of protein intrameningeally. Following this, on the 3rd day, the dose was increased to 2.0 mg. and the temperature reaction was similar to that after the original injections. The temperature rose from 101.8° before injection to a peak of 105.6° in 5 hours. A final injection of 4.0 mg. was given after 3 more days, with a rise in temperature from 102.6° before injection to a peak of 106.0° in 6 hours. The following day the temperature was normal (102.3°). 2 days after this last injection the animal appeared normal and healthy. It was killed by the intravenous injection of air. At autopsy the visceral organs were normal in the gross and on histological study. There were no hemorrhages in the tissues. The brain showed no hyperemia, edema, or hemorrhages. The dura was thickened. Supravital studies of the pia revealed a marked clasmotocytic response. The cells contained uneven sized vacuoles which varied from salmon pink to deep red. None of the typical coarse vacuole epithelioid cells, found after phosphatide, were seen. The predominant cells were lymphocytes and plasma cells, in sharp contrast to those seen after phosphatide.

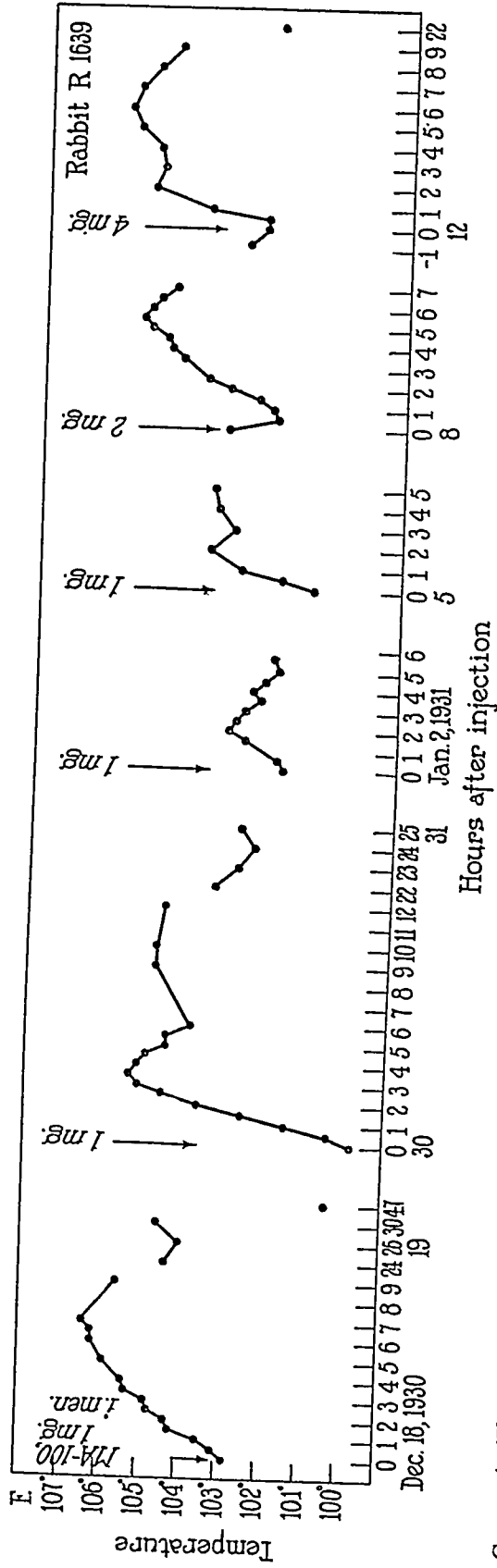


CHART 1. The changes in temperature in a normal rabbit, R 1639, after repeated intrameningeal injections, by cisternal puncture, of varying amounts of tuberculo-protein MA-100

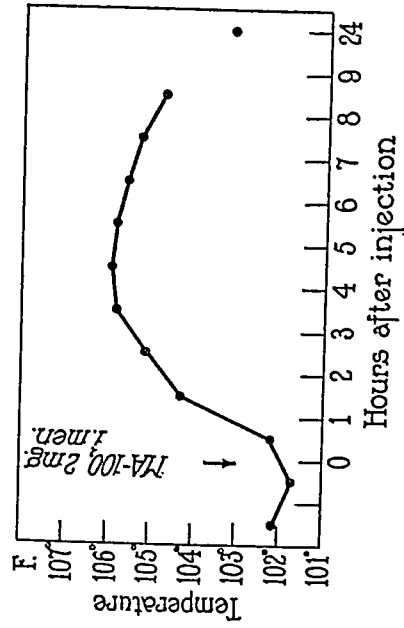


CHART 2. Fused temperatures of eight normal rabbits after intrameningeal injection, by cisternal puncture, of 2.0 mg. of tuberculo-protein MA-100.

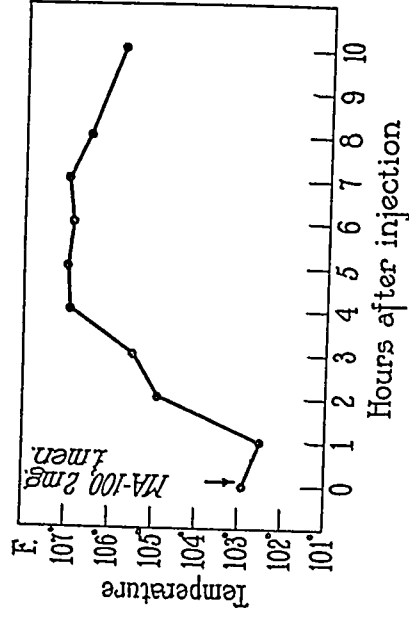


CHART 3. Fused temperatures of eight tuberculous rabbits after intrameningeal injection, by cisternal puncture, of 2.0 mg. of tuberculo-protein MA-100.

The response of the animal above was typical of the temperature reactions elicited in the normal animals. Chart 2 shows the fused temperature reactions of eight normal rabbits after a single injection of 2.0 mg. of MA-100 intravenously. The lowest peak was 105.2°, while the highest was 107.0°, in this group.

Eight tuberculous rabbits were given doses of 2.0 mg. of MA-100 intravenously. Six animals received one dose, one animal two doses, and one animal four doses. Each gave a temperature reaction and in each case of the tuberculous group, the rise was higher than that elicited in the normal animal. Chart 3 shows the fused temperature reaction after the first injection of 2.0 mg. of MA-100 in eight tuberculous rabbits. The lowest peak in the tuberculous animals was at 106.9°, and the highest 108.4° while the highest in the normal animals was 107.0°F. It would appear that the reaction to protein was more intense in tuberculous animals. One tuberculous rabbit, R 1810, after the fourth injection of 2.0 mg. of the MA-100, failed to exhibit a characteristic rise in temperature. This animal apparently had a response similar to that of the normal Rabbit R 1639. These tuberculous animals either died from the disease or were killed at varying intervals, so that the reaction was studied on the 1st, 3rd, 4th, 5th, 7th, 8th, and 30th days after injection.

Except for one animal, R 1809, which died from an accidental infection, the autopsy findings in the tuberculous animals were essentially the same at all of these periods.

The visceral organs showed advanced generalized tuberculosis. The protein is water-soluble and therefore may have passed from cerebrospinal fluid into the blood. There were no hemorrhages in any of the tissues. The brains appeared normal in the gross. There was no edema, hemorrhage, or exudate. Supravital studies of the pia showed a great increase in the number of lymphocytes and plasma cells. Typical clasmotocytes were seen in moderate numbers, but there were none of the coarse vacuole epithelioid cells such as are seen after the injection of phosphatide. A few polymorphonuclear leucocytes were present. The findings were the same in the animal killed on the day after injection of the protein, as in those killed later on.

#### *The Reaction to Tuberculo-Phosphatide A-3 and Protein MA-100 Introduced into the Subarachnoid Space in Normal Rabbits*

1 mg. of bovine phosphatide A-3 was introduced into the subarachnoid space of two normal rabbits. One animal received an injection

of 1.0 mg. of protein MA-100 in the subarachnoid space on the 6th and one on the 14th day after the phosphatide; the other received one injection of the same dose on the 14th day. There was a typical rise in temperature after each injection of the protein but otherwise both rabbits appeared normal. They were killed on the 2nd day after the last injection of protein. At autopsy the visceral organs were normal. Both of the brains appeared slightly hyperemic. No edema or hemorrhages were present. Fresh preparations of the pia on supravital study contained no monocytes or fine vacuole epithelioid cells. There was a predominance of lymphocytes, plasma cells, and cells with large vacuoles of equal size and taking a uniform stain with neutral red. This picture seemed to be a combination of that found after intrameningeal injection of phosphatide and protein.

*The Reaction to Tuberculo-Polysaccharide Introduced into the Subarachnoid Space of Normal Rabbits*

Two normal rabbits were given tuberculo-polysaccharide into the subarachnoid space. This material was prepared by the H. K. Mulford Company from the media in which the organisms had been grown. One animal received 5.0 mg. in 0.2 cc. of freshly distilled water. The animal showed a rise in temperature similar to that occurring after protein. The temperature rose from 101.3° previous to injection to a peak of 105.6° in 6 hours. The rabbit was killed 22 days later, when in excellent condition. At autopsy, the findings were normal throughout.

The other rabbit received 2 doses of 5.0 mg. in 0.2 cc. of distilled water 1 month apart and the characteristic temperature response was elicited after each injection. It remained in excellent condition and was killed 3 months after the first injection. At autopsy the tissues were normal throughout.

The polysaccharide contains a small content of nitrogen to which the rise in temperature may be due (21).

DISCUSSION AND CONCLUSIONS

From the above description one can trace the histological changes that occur in the animal during experimental tuberculous and tubercular meningitis.

The introduction of 500,000 living tubercle bacilli into the sub-arachnoid space of rabbits produces tuberculous meningitis. The disease is characterized by an incubation period of about 15 days, during which time the animal appears normal. After this period the course is progressive from weakness in the hind quarters to paralysis and death. This confirms the findings of previous investigators.

The predominant cell in the fresh preparations of the pia is one with large vacuoles of equal size which take a uniform stain with neutral red. It is significant that this type of cell appearing after injection of living or dead bacilli is the same as that seen after the injection of tuberculo-phosphatide. It is clearly a cell which has engorged itself with a great amount of one type of material, and the vacuoles appear to contain a lipid since some of the cells show myelin-like figures, which is characteristic of the phosphatide (20, 21). That it is a phenomenon of phagocytosis and not of cellular degeneration is shown by the fact that the cells are alive and active; and that the vacuoles slowly lessen in size, while at the same time their number increases, until the cell corresponds to the epithelioid cell with coarse vacuoles. In our opinion it is a young connective tissue cell, possibly less differentiated than the monocyte, which has been stimulated to marked phagocytosis of a lipid. The evidence that monocytes and younger connective tissue cells phagocytize the tuberculo-phosphatide is more convincing in the reactions of the omentum. Here it can be seen that the cells of the milk spots phagocytize the phosphatide while the clasmatoocytes of the interspaces remain relatively inactive (11). The vacuoles of the cell now under discussion are larger than those of the cell described by Sabin (20, 24) and Smithburn and Sabin (21) as the coarse vacuolated, epithelioid cell, the second stage in development of this type in the reaction to phosphatide, but they are smaller and more even in size than the first stage of the epithelioid cell as formed in the omentum and subcutaneous tissues. This cell, which is the type of the early stage of the development of the epithelioid cell, as found in the meninges, is so constant a stage in the reaction to tubercle bacilli and tuberculo-phosphatide in this area, that we have designated it the meningeal first stage of the epithelioid cell. It may be that further study will prove this cell to be of sufficient specificity to be diagnostic of tuberculous meningitis.



Lymphocytes and plasma cells increase in number after the 3rd day and soon become the most numerous cells in the meninges following the inoculation with living tubercle bacilli in the subarachnoid space. This same cellular picture is obtained after the intrathecal injection of the tuberculo-protein MA-100. The results correspond with those of Miller (25) obtained by the intraperitoneal introduction of the tuberculo-protein in rabbits.

The theory of Webb and Williams (26, 27) and of Murphy and Sturm (28) and Murphy (29) that a high percentage of lymphocytes in the blood is correlated with increased resistance of the animal has been confirmed by the more recent work in experimental tuberculosis (30, 31). But an increase of lymphocytes in the meninges is not accompanied by increased local resistance.

There has been much speculation as to the route a meningeal infection takes in spreading into the brain substance. The present experiments do not suffice for a decision on the point but the microscopic sections reveal a progressive lymphocytic infiltration of the meninges. This occurs first at the upper end of the cord near the site of inoculation, gradually extends over the midbrain and finally involves all the meningeal surfaces, invading and filling the sulci. On the 15th day there is beginning tubercle formation in these areas. At this time the vessels near the surface of the brain show perivascular lymphocytic infiltration. By the 19th day the tuberculous process in the meninges is pronounced, the perivascular infiltrations are increased, and in some areas there is lymphocytic infiltration in the brain substance. In the last stages there is extensive tuberculosis of the meninges, some of the tubercles having caseous centers. The process involves not only the meninges and sulci, but the perivascular spaces, and areas in the brain near them are extensively involved with tuberculous tissue.

Heat-killed organisms are not innocuous in the meninges. They produce lesions similar to those obtained with living organisms, but with a greatly delayed period of reaction, over 2 months. It should be said that the inoculations of heat-killed organisms were all made from the same suspension. It might be thought that a few organisms may have remained alive and propagated during this long period, until there were a sufficient number to produce the disease. Against

this is the fact that the suspension was tested for viability by inoculation into rabbits. However, the experiment is being repeated, using series inoculated both with heat-killed bacilli obtained from Dr. S. A. Petroff and with a new suspension of bovine heat-killed organisms. These experiments are not completed at the present time but the first animals of both series show the same cellular response—first stage of the meningeal epithelioid cell—as in the experiments described in this paper.

Different cell strains in the meninges apparently react to specific chemical fractions of the bacilli. The tuberculo-phosphatide stimulates the monocytic strain of cells in the meninges, causing a relatively undifferentiated monocytic cell to phagocytize this material. This cell has been described above and termed the meningeal epithelioid cell. This characteristic type of cell is produced by the living bacilli and heat-killed bacilli as well as by the tuberculo-phosphatide. The protein of the bacilli causes a rise in temperature and a proliferation of lymphocytes and plasma cells. The temperature reaction is more pronounced in tuberculous rabbits than in normal rabbits after the injection of the tuberculo-protein MA-100, though the difference is quantitative and of a small degree. It is possible that this may be an allergic phenomenon. The recent work of Sommerfeld and Zishind (32), who found little difference between the reaction in tuberculous and normal guinea pigs after the intrathecal injection of varying dilutions of tuberculin, bears out this point. Numerous investigators have referred to the importance of the rôle of allergy in tuberculous meningitis. Rich (33), states that acute tuberculous meningitis represents a model example of the allergic inflammatory-necrotizing reaction. That allergy is present in the meninges as in the other tissues of sensitive animals, is probably true. One wonders, however, if it is the most sensitive tissue to the allergic phenomenon, for if this were true one would expect after the intrathecal injection of tuberculo-protein MA-100 in a sensitive animal, a very intense inflammatory necrotic response. This did not develop in these experiments. The predominant cells in the cerebrospinal fluid and meninges after tuberculo-protein MA-100 were lymphocytes and plasma cells. A few clasmatoocytes and an occasional polymorphonuclear leucocyte were seen in the fresh preparations. None of the

epithelioid cells with coarse vacuoles such as are seen after the introduction of the phosphatide and bovine tubercle bacilli were observed after the intrameningeal injection of the protein in normal animals. The histological sections showed only a slight quantitative difference between the cellular response in sensitive and non-sensitive animals. Nowhere was there found a fulminating necrotic reaction such as one would expect if the meninges were extremely hypersensitive. Both normal and tuberculous rabbits may fail to give a temperature response to a given dose of tuberculo-protein MA-100 after repeated intrameningeal doses of the same size. However, an increase in the size of the dose in these animals elicits a temperature response similar to that of the first injection. The present experiments suggest that the number of injected organisms play a greater rôle in tuberculous meningitis, than the phenomena of sensitization.

There seems to be a definite period elapsing between the time of infection and the onset of symptoms. It is to be supposed that this period varies with the interplay of a number of factors which are common to all tuberculous infections; namely, the number and virulence of the infecting organisms, the natural or acquired immunity of the animal, and the degree of allergy present. The work of Soper and Dworski (6, 7) shows definitely that the number of infecting organisms plays a very important rôle in the length of this period.

In these experiments the injection of the various extracts from the tubercle bacilli gave characteristic cellular reactions. The dosage of these extracts was small but in every case it was many times larger than the amount of extract present in the living bacilli introduced to produce the disease. No toxic effects followed the injection of these fractions which would account for the fatal outcome of tuberculous meningitis.

#### SUMMARY

1. Both living and dead tubercle bacilli, as well as tuberculo-phosphatide, give rise to lesions in which the same type of cell is found, a cell which may prove characteristic of the meningeal reaction. After the phosphatide this cell is produced by the phagocytosis of lipoidal material. The vacuoles of these cells are slowly broken into smaller and smaller size until they correspond to the type found in

the epithelioid cells with coarse vacuoles. This change is much slower in the cells of the meninges than in the cells of the omentum and subcutaneous tissue.

2. Intrathecal injections of tuberculo-protein produce a characteristic rise in temperature in normal and tuberculous rabbits. Both normal and tuberculous rabbits, after repeated intrameningeal injections of a given dose of tuberculo-protein, may fail to give a rise in temperature and later react to a larger dose.

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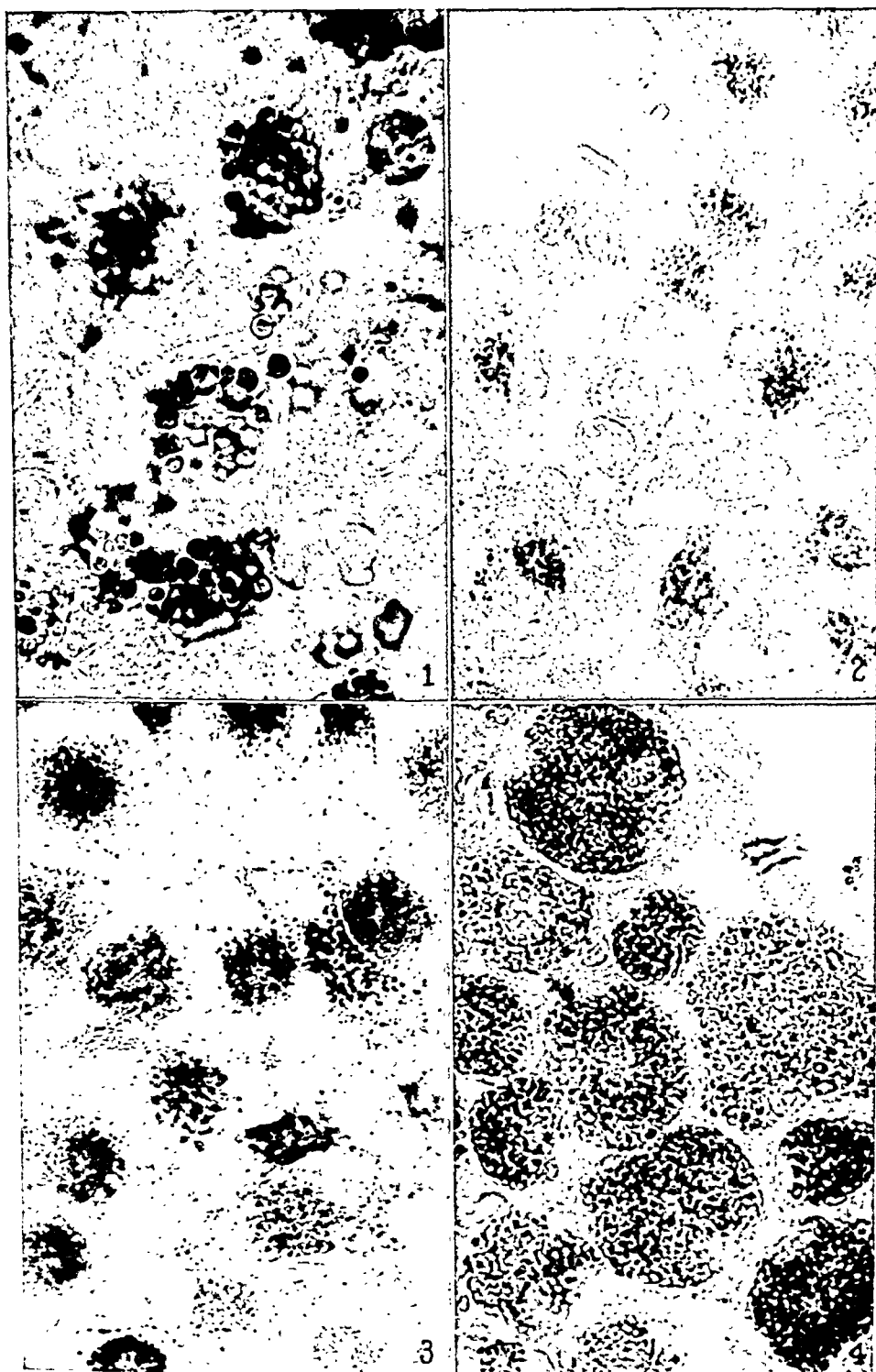
#### EXPLANATION OF PLATE 1

FIG. 1. Film of pia of Rabbit R 1782, 1 month after an intrameningeal injection of heat-killed organisms, photographed while the cells were living. Stained in vital neutral red and Janus green dye. It shows the meningeal type of cell with vacuoles of even size stained uniformly with neutral red. The reason that the vacuoles do not appear to be of even size and uniform stain is that they were all not in the same focus.  $\times 1,200$ .

FIG. 2. Scraping from over the pons of Rabbit R 2020, 6 days after it had received a half-million living bovine organisms intrameningeally, photographed while the cells were living. Stained in vital neutral red and Janus green dye. It shows monocytes, stimulated monocytes, and epithelioid cells.  $\times 1,000$ .

FIG. 3. Film of living cells in the cerebrospinal fluid taken from the fourth ventricle of R 1783 after injection of heat-killed tubercle bacilli. It shows stimulated monocytes and epithelioid cells. Stained with vital neutral red and Janus green dye.  $\times 1,000$ .

FIG. 4. Scraping from over the pons of Rabbit R 1739, 3 months after the intrameningeal injection of 2.0 mg. of bovine phosphatide. It shows the monocytic cells which have phagocytized the phosphatide, until they appear as coarse vacuole epithelioid cells. Living cells stained with vital neutral red and Janus green dye.  $\times 1,000$ .



Photographed by Louis Schmidt

(Bickford: Reactions to extracts from tubercle bacilli)



BODILY CHANGES AND DEVELOPMENT OF PULMONARY  
RESISTANCE IN RATS LIVING UNDER  
COMPRESSED AIR CONDITIONS\*

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No previous work on the toxicity of increased oxygen tensions in the respired air has been conducted under conditions in which all other factors were constantly and perfectly controlled, and most of the investigations upon this subject have been based upon data obtained from observing a limited number of animals. The possession of a large chamber, susceptible to accurate and steady adjustment, has made it possible to carry out decisive experiments on large groups of animals. The first project to which this new apparatus has been devoted consisted of observations on the life history of a standard laboratory animal living for prolonged periods in an environment normal in all respects except for the increase in the oxygen tension of the air. It was our intention to determine whether or not any acclimatization could be produced against the toxic action upon the lungs of high oxygen tensions and, if so, what changes in the animal were responsible for this adaptation. The data we have accumulated deal with a single species, the standard albino rat, bred from Wistar Institute stock, exposed to compressed air having an oxygen tension of 635 mm. of mercury equivalent to 83.6 per cent oxygen at normal barometric pressure.

EXPERIMENTAL

*A. The High Pressure Chamber.*—A detailed description of the construction, operation, and experimental capabilities of the apparatus used in this research has been published by Thomson (1). The pressure chamber consists of a  $\frac{1}{2}$  inch thick steel cylinder, 31 feet long and 8 feet in diameter. It is divided into three

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\* Publication No. 2, from the Miriam Smith Rand Fund.



compartments, each of which may be used independently of the others. The center compartment is the smallest and is known as the lock. By means of it one may enter and leave the high pressure end compartments in which the animals are kept, without altering the conditions of their environment. Two electrically driven air compressors located in an adjoining room supply air at the desired pressure and volume flow to the pressure chambers. After leaving the compressors the air passes through an automatically controlled air-conditioning apparatus where it is scrubbed and brought to predetermined dry and wet bulb temperature readings. Continuous written records of the air pressure and dry and wet bulb temperatures were obtained automatically during the entire experiment.

*B. Conditions of the Experiment.* 1. *Barometric Pressure.*—Increased oxygen tension and increased barometric pressure were the two possible abnormal factors in these experiments. Many authors (2, 3,<sup>1</sup> 4–9) have presented concise evidence that the increased oxygen tension, and not the barometric pressure, was responsible for the symptoms of poisoning found under such conditions. The results of our experiments have confirmed these earlier observations, and therefore it may be assumed that the only factor varying from the normal in our experiments was the oxygen tension. The barometric pressure in the experimental chamber was maintained at 3040 mm. Hg for 30 days in the first experiment. In the second, this same pressure was held for 72 days except for approximately 1 hour each day, when it was reduced to 2280 mm. Hg while observers entered the animal chamber. This was done in order to lessen the time necessary for the decompression of attendants from 82 minutes to 32 minutes. The results of each experiment were identical, showing that this transient variation had no effect upon the animals. The oxygen tension, therefore, was approximately 635 mm. Hg or 83.6 per cent of an atmosphere, except for the temporary daily drop to 60 per cent during the second experiment.

2. *Temperature.*—The dry bulb temperature was kept at 28°C. plus or minus 1°C. This was found to be the temperature at which the animals were most active and apparently most contented. Benedict and MacLeod (10) have showed this to be the critical temperature for the albino rat, as the metabolism becomes basal at this reading.

3. *Humidity.*—The relative humidity ranged from 49 to 50 per cent during all the experiments.

4. *Volume Flow.*—Conditioned outdoor air was circulated through the animal chamber at the rate of 2660 liters per minute. The composition of the air was found to be practically identical to that of outdoor air on numerous gas analyses. This rate of ventilation was also sufficient to keep the odors of animal excretions from accumulating in the chamber.

5. *Duration of Exposure.*—The first group of animals was exposed continuously for 30 days. A second group was exposed for 72 days, and then after an interval of 40 days at normal pressure was reexposed for 10 more days.

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<sup>1</sup> Bert (3), pp. 611–612.

6. *The Albino Rat*.—The albino rat is a standard laboratory animal whose life history is accurately known. Although sporadic cases of pulmonary infection are found, they occur in a relatively small percentage of animals. The life cycle is completed rapidly, and it is therefore possible to observe the reaction of the animal as it passes from adolescence to maturity.

7. *Number of Animals Employed*.—At the beginning of these experiments 147 animals of representative age groups and sexes were selected for observation. Twenty-one of these were killed and autopsied to give normal tissue sections to serve as controls for a pathological study. During the course of the experiments 97 rats were born and observed for varying periods of time under the prevailing experimental conditions, making a total of 244 animals studied.

8. *Care of Animals*.—All rats were cared for in the same manner. Two standard diets were employed, each being used alternately.<sup>2</sup> Daily feedings were maintained at all times except on Sundays and holidays. In addition to the standard diets lettuce and fresh whole milk were given daily. 20 to 30 cc. of cod liver oil were added per liter of milk twice a week. No more than eight rats were housed in a single cage, 2 feet square and 1 foot high. A certain number of cages was changed daily, so that all animals had clean quarters at least once a week.

<sup>2</sup> *Standard Rat Diets*.—

No. 1. Fairhall, L. T., *Am. J. Physiol.*, 1928, 84, 382.

Wheat flour.....	per cent
Corn meal.....	35.3
Rolled oats.....	23.5
Dextrin.....	23.6
Powdered liver.....	11.7
	5.9
	<hr/> 100.0

Add 10 gm. of calcium phosphate to each kilo of food. Make into a dough, then roll and cook into biscuits.

No. 2. Greenman, M. J., and Duhring, F. L., *Breeding and care of the albino rat for research purposes*, Philadelphia, Wistar Institute, 1923, 51.

Lima beans.....	per cent
Rice.....	19.0
Whole wheat.....	27.0
Rolled oats.....	27.0
	27.0
	<hr/> 100.0

Add water and cook until well done. Allow excess moisture to evaporate, then pour on large trays. It may be kept for many days in a cold room.

A liberal amount of wood wool was supplied to absorb the moisture and provide nesting material. Especial care was taken in the handling of the animals. They were fondled daily and were absolutely gentle during all observations. Rats are exceptionally responsive to this kind of treatment, and their behavior served as an important index of the general well-being. If in good health all animals clung to the front of the cages when observers entered the chamber. They displayed intense interest in the feeding procedures and daily weighings. When ill all such interest disappeared, and the animals became markedly apathetic, failing to respond even when the cages were opened.

9. *Accumulation of Data.*—Weighings were made under pressure on a direct-reading, Toledo, springless balance. Two light aluminum boxes were constructed to hold about ten rats, so that all in one cage could be weighed at once, thus eliminating individual weighings. A small number of animals were weighed individually. Daily notes were made regarding the condition of the rats, and any dead or obviously dying animals were removed for autopsy. The details of the pathological findings are reported in a second publication (11).

In order to avoid caisson disease the stage decompression table compiled by Boycott, Damant, and Haldane (12) for the British Admiralty was followed. Neither observers nor animals showed the slightest evidence of nitrogen bubble formation.

### *The Effect of the First Exposure to Increased Oxygen Tension*

A. *Acute Oxygen Poisoning.*—Many authors (2, 4, 5, 7, 8, 11, 13, 14–18) have showed that the damage from acute poisoning by oxygen in concentrations such as we have provided is centered in the lungs. Active hyperemia and pulmonary edema dominate the picture. The significant fact brought out by our observations is the comparative immunity of young rats to an oxygen concentration which invariably causes serious illness or death in adults. By young rats we refer to animals from birth to 3 months of age. After the 3rd month rats still display a degree of the resistance to high oxygen concentrations which is characteristic of early life, but this quality diminishes steadily and by the 6th month is gone. Faulkner and Binger (19) found that young turtles were less susceptible to oxygen poisoning than were adults, but the differences reported were not so definite as we have found in rats.

1. *Old Rats (over 3 Months of Age).*—The animals lived in the experimental chamber under normal atmospheric conditions for 5 days prior to compression. They were in excellent condition during this period, and their weight curves showed a normal upward trend

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(Text-fig. 1). On the 6th day the air pressure was gradually raised to 3040 mm. Hg within an hour, and remained at this point for 30 days in the first experiment and 72 days in the second. The results of both experiments were identical, and for the sake of simplicity they will be considered together in the presentation of data.

All rats were in excellent health until the 3rd day of exposure. At this time several were found to be weak, apathetic, and dyspneic. They showed no interest in the feedings. The majority, however, were still in good health. The weight curves showed that since the beginning of exposure the normal growth rate had decreased in some of the groups of animals (Text-fig. 1, Curves 2, 3, and 4).

During the 4th day the symptoms of acute oxygen poisoning became marked in all, and the characteristic pathological findings were present (11). There was an average loss of 10 to 15 gm. in weight from the 3rd day. The rats were acutely ill, and showed extreme respiratory embarrassment and hyperpnea. Various degrees of cyanosis were present. There was practically no food consumption during the preceding 24 hours (Text-fig. 3, Curve 2), and the mortality rate was 13 per cent, all the deaths occurring on the 4th day (Table I).

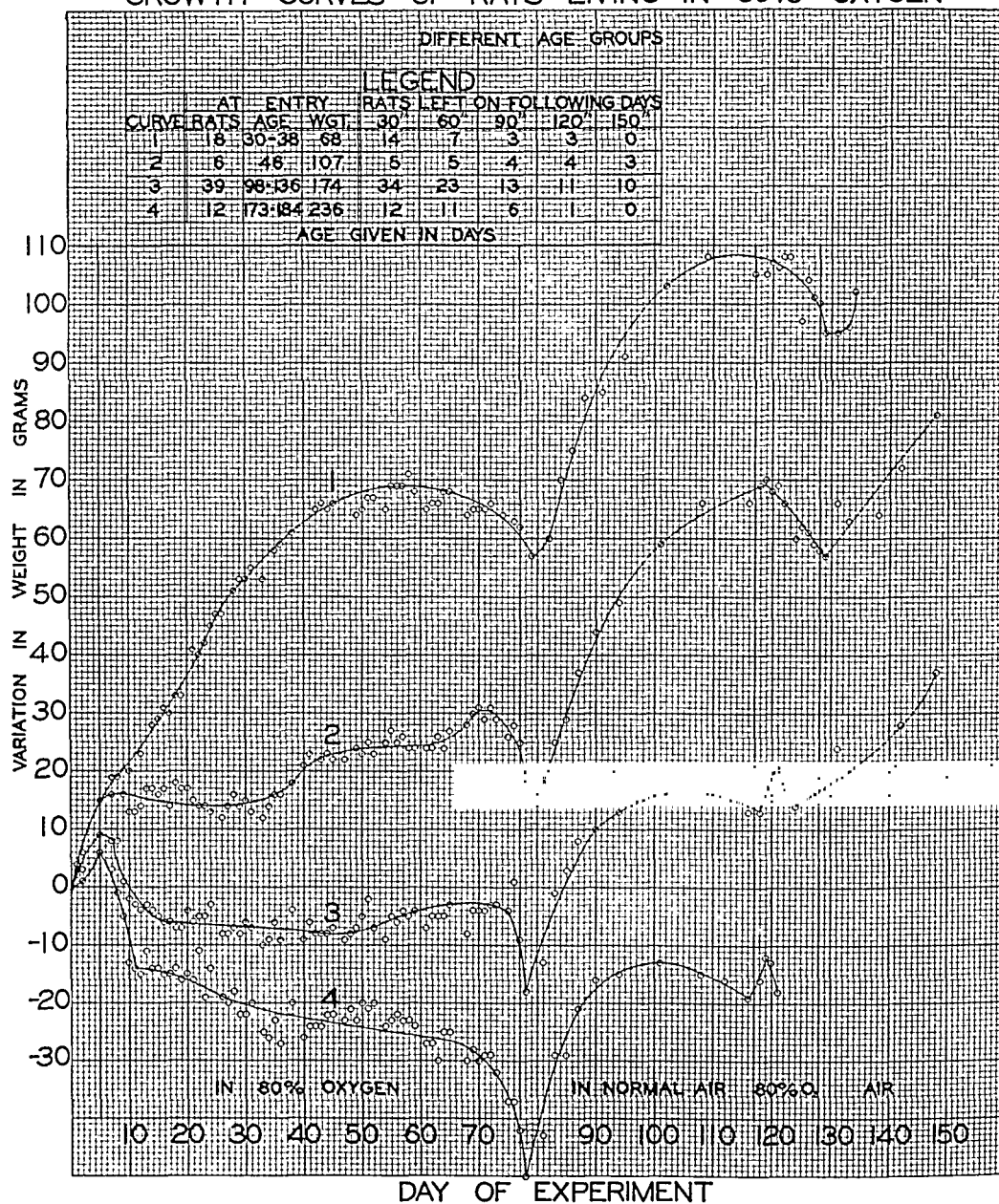
On the 5th day most of the surviving rats were in good condition but had continued fasting during the preceding 24 hours (Text-fig. 3, Curve 2).

By the 6th day all rats were in excellent health objectively, and the pathological changes found on the 3rd and 4th days had almost disappeared. They were eating again, but the food consumption was only half the amount prior to exposure, and the original loss in weight persisted with slight fluctuations.

2. *Young Rats (under 3 Months of Age).*—These animals did not show objective signs of acute oxygen poisoning at any time during exposure. They continued to be exceedingly active and playful, and their appetite was good. In contrast with old rats they showed no loss in weight, yet there was a definite depression of the normal growth rate.

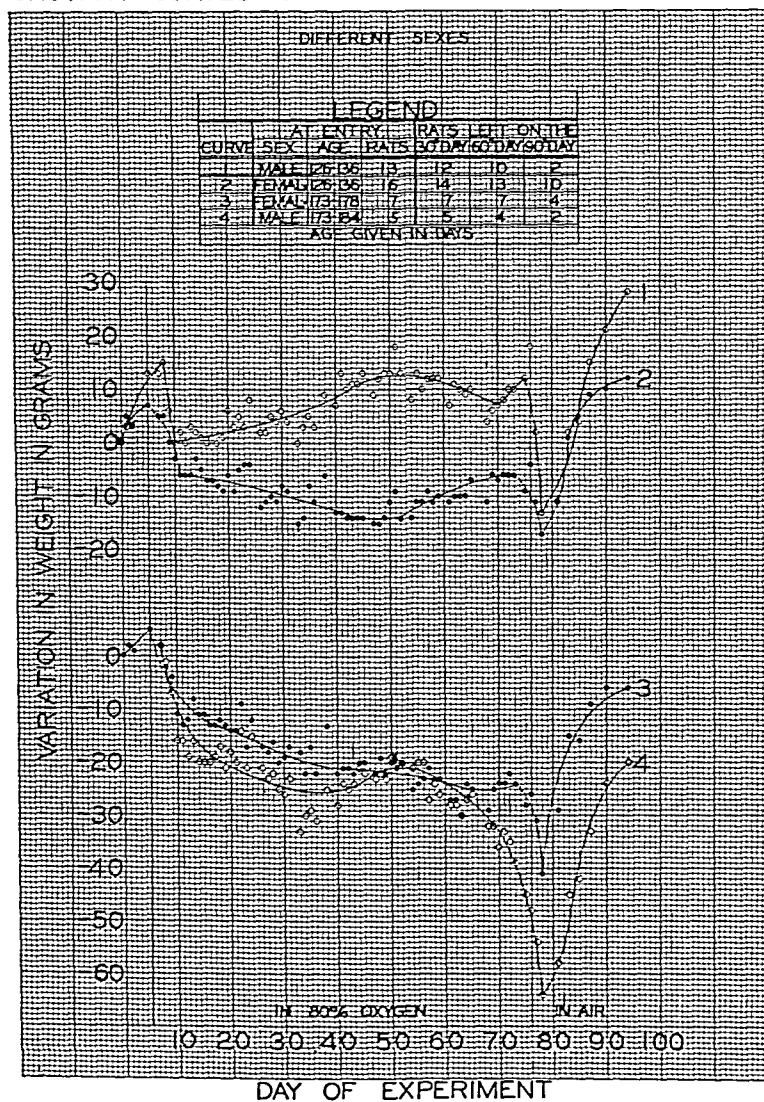
Young adult rats from 100 to 150 days of age reacted in an intermediary manner to that described above. Symptoms of illness were observed during the acute stage of pulmonary edema on the 3rd and 4th days, but the animals remained in much better condition than

## GROWTH CURVES OF RATS LIVING IN 80% OXYGEN



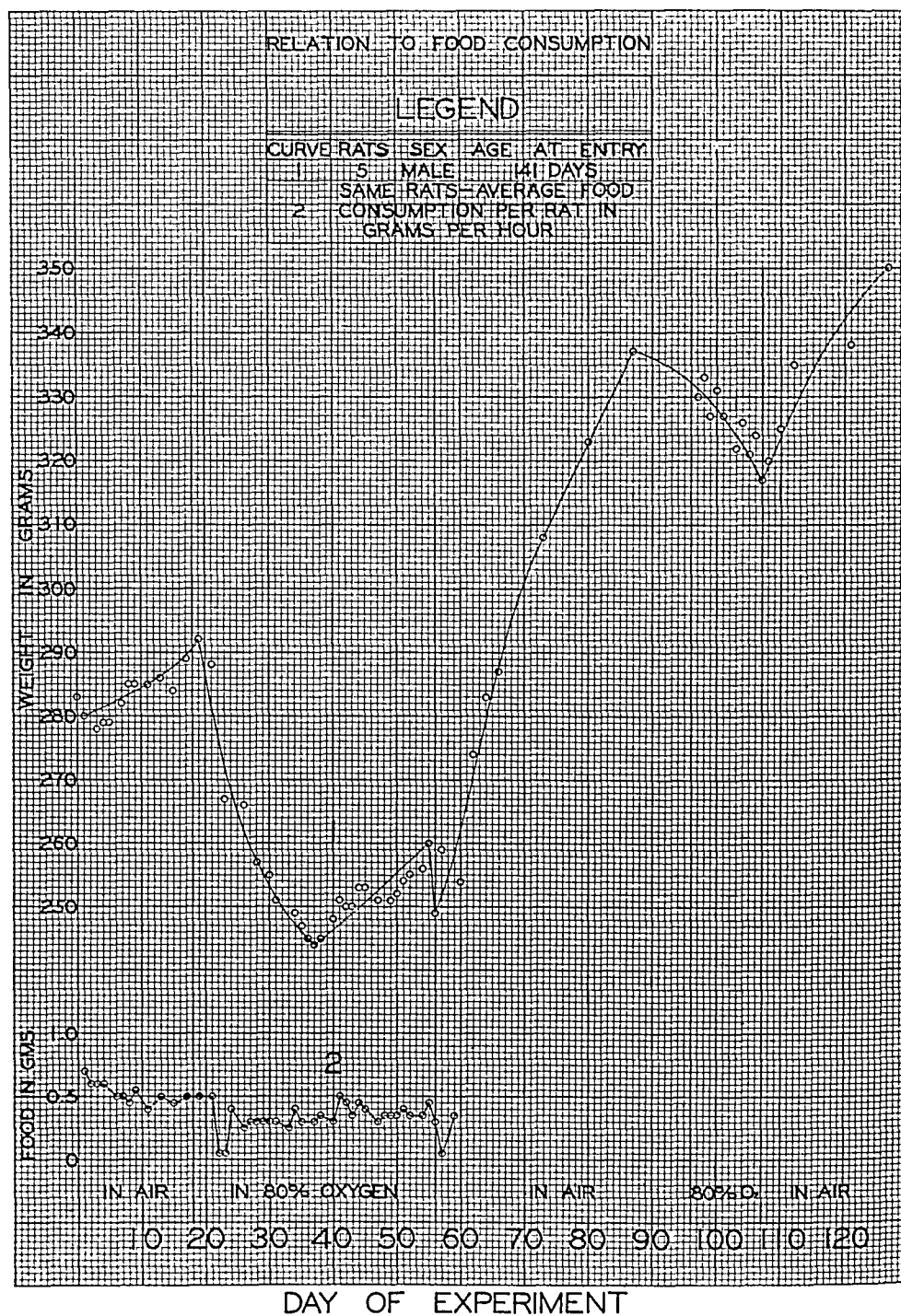
TEXT-FIG. 1. The relationship of age to the development of acute oxygen poisoning, showing the increased susceptibility of rats with advancing age.

## GROWTH CURVES OF RATS LIVING IN 80% OXYGEN



TEXT-FIG. 2. The relationship of sex to the development of acute oxygen poisoning, showing that both sexes react in essentially the same manner.

## GROWTH CURVE OF RATS LIVING IN 80% OXYGEN



TEXT-FIG. 3. The relation of growth curves to food consumption, showing a decrease in appetite.

older rats. There was less loss in weight and a much lower mortality rate (Text-fig. 1, Curve 3; Table I, Group 4, 4th day of exposure).

The difference in the effect of 83.6 per cent oxygen on old and young animals can be correlated with a difference in lung structure (11). This difference is essentially a far greater cellularity of the alveolar walls in young rats.

*B. Prolonged Exposure.*—During the 2nd week all rats continued in good condition, although the weight of the older ones remained at the low level established on the 4th day of exposure (Text-fig. 1, Curves 2, 3, and 4). The young rats were gaining in weight (Text-fig. 1, Curve 1). The food consumption continued to be about half the amount prior to exposure (Text-fig. 3, Curve 2).

Sporadic cases of illness appeared early in the 3rd week in old rats. The animals affected had a poor appetite, lost weight rapidly, became dyspneic, apathetic, inactive, weak, and eventually died of a bronchopneumonia. The incidence of this disease was practically the same as that of the sporadic respiratory infection found in supposedly normal rats, although in the latter it did not progress further than lobar atelectasis and purulent bronchitis (11). Cases of chronic bronchopneumonia were found during the remainder of exposure in roughly 22 per cent of the rats. Eleven died at various times without showing any evidence of bronchopneumonia; in all these there was considerable fibrosis in the lungs apparently referable to the acute experience suffered on the 3rd and 4th days. The final cause of death in these animals cannot be given.

With the exception of the animals dying as has been described, the rats remained in good health after the 1st week, but they were not entirely normal. Food consumption and weight were low, indicating that although nothing seriously wrong could be detected on daily inspection, some definite influence was operating steadily to keep them in a slightly subnormal state. Such animals, when killed and examined microscopically, always showed changes in the lungs—most characteristically an increased cellularity, so that the lungs resembled those of young animals (11).

On the 72nd day the exposure was discontinued. During decompression or within 24 hours afterwards, thirteen, or 28 per cent, of the rats surviving the 72 days exposure died (Table I). All showed marked degrees of lung damage, and in the majority it was due to bronchopneumonia. No paralyses were observed, and none of the



deaths could be attributed to caisson disease. The animals that lived through decompression were in good health and showed no bronchopneumonia at subsequent autopsies (11).

A temporary loss in weight followed removal to normal air. During the next few weeks a great increase in growth occurred, being most rapid the first few days after the conclusion of exposure (Text-figs. 1 to 3). The gain in weight averaged 40 to 50 gm.

#### *Adaptation—Reaction during Second Exposure to Increased Oxygen Tension*

All rats surviving the 72 day period in 83.6 per cent oxygen were kept in normal air for 40 days. They were then reexposed for 10 days to the previous conditions of high oxygen concentration. No acute change in health occurred during this time, although a slow loss in weight was seen. There were no signs of acute oxygen poisoning either clinically or pathologically (11). Five control rats, for which this was the first exposure, developed typical signs of oxygen poisoning beginning on the 3rd day. Unquestionably, during the first exposure the animals had become resistant to the toxic effect of this environment. Nine litters were born during the first exposure and all died. One litter was born in the second period of exposure, and six out of the eight young survived and developed normally. This was further evidence that some permanent adaptation had been produced. On removal to normal air all rats remained in excellent health, contrary to the outcome at the end of the first exposure when there was a mortality of 28 per cent. At the present writing, 214 days after the conclusion of this experiment, the survivors are in excellent condition.

#### *Mortality Statistics*

The mortality rates for males and females of different age groups have been compiled in Table I. Since a large number of rats were killed at different times during exposure, it was impossible to obtain accurate data regarding the mortality for the entire period. Nevertheless, an approximation has been reached by dividing the total number of deaths during exposure and decompression by the sum of the number of rats surviving the experiment plus the number dying during the experiment.

Table I shows that all deaths from acute oxygen poisoning occurred on the 4th day in 80 per cent oxygen. There were no deaths in rats

TABLE I

*Mortality Statistics for Different Age Groups and Sexes after Varying Periods of Exposure to 83.6 Per Cent Oxygen Tension*

Day of exposure.....		1st					2nd					3rd					4th				
Age group.....		1	3	4	5-6	Total	1	3	4	5-6	Total	1	3	4	5-6	Total	1	3	4	5-6	Total
No. of rats exposed	M	12	1	26	19	58	12	1	25	19	57	12	1	24	18	55	12	1	23	17	53
	F	32	4	26	15	77	32	4	25	15	76	32	4	24	15	75	32	4	23	15	74
	MF	44	5	52	34	135	44	5	50	34	133	44	5	48	33	130	44	5	46	32	127
No. of rats dying	M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	4
	F	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	2
	MF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	4	6
Per cent mortality	M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	18	7
	F	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	7	3
	MF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	13	5

Day of exposure.....		5th					During decompression					During all experiment				
Age group.....		1	3	4	5-6	Total	1	3	4	5-6	Total	1	3	4	5-6	Total
No. of rats exposed	M	12	1	21	14	48	3	1	7	4	15	4	1	9	7	21
	F	31	4	21	14	70	7	2	12	10	31	10	2	15	12	39
	MF	43	5	42	28	118	10	3	19	14	46	14	3	21	19	57
No. of rats dying	M	0	0	0	0	0	1	1	3	2	7	2	1	6	5	14
	F	0	0	0	0	0	2	0	2	2	6	5	0	5	4	14
	MF	0	0	0	0	0	3	1	5	4	13	7	1	11	9	28
Per cent mortality	M	0	0	0	0	0	33	100	43	50	47	50	100	67	71	67
	F	0	0	0	0	0	29	0	17	20	19	50	0	33	33	36
	MF	0	0	0	0	0	30	33	26	29	28	50	33	52	47	50

M, male, F, female. MF, male and female. Age group, approximate age in months.

less than 120 days old, while in older ones the number of deaths was proportional to the advance in age. There was no significant difference in the mortality rate for males and females.

*Effect of Pregnancy*

Six pregnant rats in the late stage of gestation were exposed with the others at the beginning of the experiment. Three of these were 9 months of age, and all died of acute oxygen poisoning, one as early as the 2nd day. The other three pregnant rats were 130 days old, and two of them died. The third survived and gave birth to a normal

TABLE II

*Mortality Rate and Average Weight of Litters Born during and after Exposure of Mothers to 83.6 Per Cent Oxygen Tension*

No. of litters	Total No. of rats in litters	No. dying within 24 hrs. after birth	No. surviving	Mortality	Total weight of litters	Average weight per rat
Litters born in tank during first exposure of mothers						
3	16	All	0	<i>per cent</i> 100	<i>gm.</i> —	<i>gm.</i> —
1	5	All	0	100	14.7	3.34
1	8	All	0	100	25.6	3.20
1	9	All	0	100	32.4	3.60
6	38	All	0	100	72.7	3.30
Litters born within 24 hrs. after decompression of mothers						
2	12	All	0	100	—	—
1	8	All	0	100	29.9	3.70
3	20	All	0	100	29.9	3.70
Litters born 6 to 39 days after decompression of mothers						
4	31	2	29	6.5	—	—
Litters born in tank during second exposure of mothers						
1	8	2	6	25	—	—

litter after she was removed from the high oxygen. Apparently rats advanced in pregnancy cannot survive acute oxygen poisoning as well as non-pregnant animals of the same age. The encroachment upon the thoracic cavity by the enlarging fetuses is probably the cause of this increased susceptibility.

Rats entering 80 per cent oxygen in the early stages of pregnancy,

as well as those becoming pregnant during exposure, were as resistant to acute oxygen poisoning as non-pregnant animals. The litters, however, were never carried to term under these conditions. Table II shows the average weights of litters born during the experiment. Each survived for a few hours, but according to the birth weight given for normal rats by the Wistar Institute these litters were almost 50 per cent underweight. Nine litters were born during the first exposure of the mothers, and all died within 24 hours after birth.

During the second exposure an acclimatized female gave birth to a litter on the 4th day, and six of the eight young survived. The mother remained in excellent health. This is a striking example of the difference in reaction of adapted animals, because during first exposure all the litters, and almost 100 per cent of the mothers advanced in pregnancy died. Furthermore, this litter was born on the 4th day of exposure when the symptoms of acute oxygen poisoning are most intense in non-adapted rats (Table II).

The fertility of rats was not altered by prolonged exposure. Females living with males throughout the exposure had frequent litters.

#### DISCUSSION

Previous investigators have failed to demonstrate any acclimatization or adaptation of animals to toxic oxygen tensions (16, 20). Although there may be several reasons for this, the most important is probably the fact that definite damage to the animal was not produced during the first exposure, so that there was no stimulation to the lungs or other defensive mechanisms of the body. Barach (20) kept rabbits in 60 per cent oxygen for a prolonged period in an attempt to produce acclimatization and then raised the oxygen tension to toxic heights, but oxygen poisoning developed in a typical manner. The exact border-line of toxic oxygen tensions has not been established, although the available evidence (2, 3,<sup>3</sup> 4, 13, 20) suggests that it lies around 70 per cent of an atmosphere. Species variation to this toxicity may be expected, so that it can be assumed that 80 per cent oxygen is just at the border-line of dangerous toxicity for the albino rat. Whereas higher tensions might prove fatal during the acute

<sup>3</sup> Bert (3), pp. 611 and 844.

stage of oxygen poisoning, most rats are able to combat successfully the acute pulmonary edema produced by 80 per cent oxygen with eventual complete removal of exudate. The lung damage or stimulation leads to distinct changes in morphology (11) which persist for at least 53 days after returning to normal air.

The significance of these alterations in the adaptation occurring in old rats and the difference in the reaction of young rats to acute oxygen poisoning is the subject of a second paper (11) to which we have frequently referred.

The gradual loss in weight, even though all signs of acute oxygen poisoning have disappeared, is conclusive evidence that the normal physiological processes are constantly altered during exposure. No satisfactory pathological changes were found in the first 30 days of exposure to account for this weight loss in animals remaining otherwise normal. After the 1st month of exposure the presence of chronic vascular lesions in the lungs (11) was confirmatory evidence of the toxicity of this environment, although no clinical changes in health could be detected. The effects produced by prolonged exposure to toxic oxygen tensions should be termed "chronic oxygen poisoning" in contradistinction to the pulmonary edema associated with acute oxygen poisoning. On removal to normal air the rapidity of growth indicated that the inhibitory effect of the previous environment quickly disappeared. The loss of weight during a second exposure in the absence of any clinical or pathological changes was again evidence of this alteration in the normal physiological processes. A certain number of determinations of basal metabolism were made upon animals under continuous exposure to high oxygen. While the subject should be explored further, it is safe to say that the metabolism was not increased, so the loss of weight may consequently be ascribed to loss of appetite, the final reason for which cannot be given.

The steady though subnormal gain in weight in young rats is probably in part due to greater resistance to oxygen poisoning and in part to a greater stimulus for growth in these than in adults, so that growth progresses in spite of the adverse environment.

#### SUMMARY AND CONCLUSIONS

1. 244 albino rats from standard Wistar Institute stock have been kept for periods up to 72 days under the following conditions.

(a) Barometric pressure: 3040 mm. Hg. This means a partial pressure of oxygen of 635 mm. Hg and is equivalent to an 83.6 per cent oxygen mixture at normal barometric pressure.

(b) Temperature: 28°C.

(c) Humidity: 50 per cent relative.

(d) Rate of ventilation: 2660 liters per minute for all animals.

(e) Food and daily care were provided which induced normal growth in rats in the usual laboratory quarters.

2. The harmful factor in this environment was the increased oxygen tension. In our experiments the acute effects were active hyperemia and edema of the lungs, just as have been described by many investigators.

3. Only a small percentage of rats die from acute oxygen poisoning at the pressure employed. The majority return to good health objectively and survive several months of exposure.

4. Rats under 1 month of age display no clinical signs of acute oxygen poisoning, while in older animals the severity of the reaction and the mortality is directly proportional to the advance in age.

5. The symptoms of acute oxygen poisoning appear on the 3rd day of exposure in adults and reach maximum intensity during the 4th day, all deaths occurring at this time.

6. A continued weight loss is found in old rats, while the young gain weight, but not with normal vigor.

7. Adaptation to this toxic oxygen tension occurs in the albino rat during the first exposure, so that on reexposure acute oxygen poisoning does not develop.

8. Respiratory infection occurred sporadically in roughly 20 per cent of the normal adult rats in our laboratory colony. About the same incidence was found in the experimental rats during exposure; in most of these, chronic bronchiectasis and bronchopneumonia followed and proved fatal, indicating a lowered resistance in exposed animals.

9. Most rats exposed to an 80 per cent oxygen tension late in pregnancy have premature litters and die of acute oxygen poisoning, but if exposed early in pregnancy the majority survive.

10. Litters born during the first exposure of the mother are approximately 50 per cent underweight and die during the first 24 hours after birth.

11. A female, rendered resistant by a first exposure, produced a healthy litter during second exposure.

12. The continuous failure of adults to gain in weight and the fact that young animals grow slowly, together with the slowly progressing pulmonary pathology (11), indicate that high oxygen tensions not only produce acute changes in the lungs but also some alteration in the normal physiological processes, which may be termed "chronic oxygen poisoning."

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# MORPHOLOGICAL CHANGES IN THE LUNGS OF RATS LIVING UNDER COMPRESSED AIR CONDITIONS\*

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PLATES 2 TO 5

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Previous papers (1, 2) have described the apparatus and technique used by the authors in prolonged experiments upon the effects of high oxygen tensions on rats. The structural changes produced in the lungs of animals living in an environment having a high oxygen tension, particularly those accompanying long exposure, have never been thoroughly observed, nor has the effect of age received any appreciable comment. Our observations have shown that rats under 1 month of age do not give clinical evidence of suffering the acute pulmonary effects of 83.6 per cent oxygen; that these effects appear with advancing age; that older animals surviving acute oxygen poisoning are immune on second exposure. In this paper we describe structural differences in the lungs of young and old rats; differences in the effects of 80 per cent oxygen on animals of various ages; and finally the structural changes found in the lungs of rats which have become resistant to the acute effects of toxic concentrations of oxygen.

## *Autopsies and Pathological Technique*

244 albino rats were observed during this study. 137 autopsies were performed, 21 of which were upon normal animals to serve as controls. All dead and dying animals were removed for autopsy as soon as possible. In addition to these, live animals were removed every day during the first 8 days of exposure, and at weekly or biweekly intervals after that. Live rats were killed with an intraperitoneal injection of 0.5 to 1.0 cc. of 1 per cent potassium cyanide. The lungs were removed and fixed in Zenker's fluid for 24 hours. Hematoxylin and eosin stain was used

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routinely. In addition to this, Mallory's aniline blue stain for connective tissue, Mallory's phosphotungstic acid hematoxylin stain, and Foot's reticulum stain were made on the lungs of representative animals after varying periods of exposure.

### *Histology of the Normal Rat Lung*

Grossly the lungs of old and young rats are a diffuse, uniform pink color, and no distinction exists other than the difference in size. Microscopically a striking difference in the cellularity of the alveolar walls is found.

From birth to roughly 4 months of age the alveolar walls are much more cellular and thicker than in adult animals. The individual alveolar cells in young animals are larger and more distinct than in adults, and the nuclei are more vesicular. This cannot be explained on the basis of incomplete expansion of the lungs, because the alveolar walls in areas having equivalent distention in young and old still show this prominent difference in cellularity (Figs. 1 to 4).

During the 5th and 6th months of life the size and number of the alveolar lining cells gradually diminish. After 6 months of age the microscopic appearance is constant. The alveolar walls are thin and contain relatively few lining cells, the nuclei of which stain more intensely than in young rats. The difference between the lungs of rats under 100 days of age and those in the 6th or 7th month of life is so distinct that one can invariably distinguish young from old by study of lung sections (Figs. 1 to 4).

### *Pathology of Acute Oxygen Poisoning at Different Ages*

*A. Adult Rats (over 5 Months Old).*—The pathological changes in acute oxygen poisoning are essentially those of an intense pulmonary edema, as first described by Lorrain Smith (3, 4) and confirmed by numerous authors, with right-sided dilatation of the heart as described by Karsner (5). The other organs of the body are normal except for congestion of the viscera and a slight amount of tubular damage in the kidneys. This injury was manifested by areas of degeneration and the presence of occasional mitotic figures in the tubular epithelium.

*Gross Pathology.*—The first evidence of the toxicity of 80 per cent oxygen tensions for rats appears during the 3rd day of exposure and becomes most intense on the 4th day. A description of the clinical picture has been given in a previous paper (2). The normal, uniform pink coloration of the lungs is replaced by a diffuse mottling with dark beefy red, and purple patches due to the intense congestion. Occasionally small white spots of peripheral emphysema are found. The lungs are greatly distended with edema and occupy almost the entire thoracic cavity. When the latter is opened, they fail to collapse. There is a pleural

effusion of several cubic centimeters of clear, pale yellow, serous fluid which clots on standing. The quantity averages about 5 cc. on the 3rd day, but on the 4th day it is increased to approximately 10 cc. This effusion in itself is probably one of the greatest causative factors for the respiratory embarrassment observed.

*Microscopic Findings.*—Most of the alveoli are filled with an eosinophilic, finely granular, serous exudate, containing a number of desquamated alveolar cells, polymorphonuclear neutrophiles, red blood cells, and fibrin (Figs. 6 and 7). Occasionally there is a slight to moderate amount of patchy hemorrhage into the alveoli. Many desquamated alveolar cells are present.

Considerable emphysema is found in the alveolar ducts and alveoli toward the periphery of the lungs, and is most marked in rats showing the greatest amount of pulmonary edema.

The capillaries of the alveolar walls are engorged with red blood cells. The arteries and veins are surrounded by a large, loose meshed, clear zone of perivascular edema into which there are varying degrees of cellular infiltration. This gives a very striking picture under low power magnification (Figs. 5 and 7). The cellular infiltration consists mostly of large mononuclear phagocytes. Polymorphonuclear eosinophiles and neutrophiles are present in lesser numbers, the former being more numerous. Cells of the lymphocytic series are occasionally seen. Numerous perivascular lymphatics are found throughout the lungs. They are dilated and contain precipitated serum. These are increased above normal in size and numbers (Fig. 7). The alveolar capillaries and walls contain a relative increase in the number of polymorphonuclear leucocytes (Fig. 6). The trachea, bronchi, and bronchioles are uninjured.

*B. Young Rats (under 100 Days of Age). Gross Pathology.*—In rats from 9 to 40 days of age there are no abnormal findings. Rats 100 days old at entry show definite signs of congestion on the 4th day, although the edema is very slight as compared with older animals. In some cases there is a slight pleural effusion.

*Microscopic Findings.*—Rats under 40 days of age show no evidence of oxygen poisoning other than an early perivascular edema and slight cellular infiltration with some dilatation of lymphatics (Fig. 5). Animals approximately 100 days of age show marked perivascular edema and cellular infiltration, dilated lymphatics, some desquamation of alveolar cells, but only small patchy areas of alveolar exudate as compared with older animals. The hyperplasia and hypertrophy of the alveolar lining cells is marked.

#### *Mortality Statistics for Different Ages and Sexes*

An analysis of the mortality data has already been published (2). It suffices to say here that all deaths from acute oxygen poisoning

occurred in rats on the 4th day of exposure. There were no deaths in animals under 120 days of age, while in the older animals the mortality was directly proportional to the age. There was no significant difference in the mortality rate for males and females.

### *Changes in the Lungs of Surviving Animals during Continued Exposure*

*Findings from 4th through 7th Days.*—During the 5th and 6th days of exposure there is a rapid removal of the serous exudate from the alveoli. After the 5th day no animal showed any pleural effusion. By the end of the 1st week of exposure all evidence of the acute edema which began on the 3rd day had disappeared, and the lungs resumed a more normal gross appearance. The dark purple color previously seen, changed gradually to a dark red, and then to mottled pink about the 7th day. These color changes paralleled the disappearance of the generalized congestion such as was seen microscopically. The lungs did not reacquire a diffuse, pink coloration but became finely mottled with small, pink areas on a white or greyish white background, some of this latter being due to small blebs of emphysema. The mottling persisted throughout the experiment and even after the animals had been returned to normal air for as long as 214 days. Numerous areas of decreased blood supply were found microscopically, in which it was difficult to distinguish the capillaries because of the few red blood cells present. This gross mottling of the lungs is explicable on the basis of the irregular distribution of blood through the capillary bed.

Microscopically the lungs were in fair condition. The alveolar exudate had disappeared, the perivascular edema and cellular infiltration were decreased and in some instances gone. The alveolar cells, on the other hand, were enlarged and increased in number causing a marked thickening of the alveolar walls. A slight amount of desquamation persisted. Areas of emphysema were present, but more striking were the small patches of atelectasis, involving only a few alveoli irregularly throughout the lungs.

### *Animals Developing Secondary Respiratory Infection*

At the end of the 3rd week of exposure a few of the older animals began to die at sporadic intervals. At autopsy, all of these showed varying degrees of purulent bronchitis, bronchiectasis, and bronchopneumonia. Such rats had a poor appetite, lost weight rapidly, and became dyspneic, apathetic, inactive, and weak. Grossly the lungs showed congestion, consolidation, and frequently lobar atelectasis, while microscopically the characteristic findings of bronchopneumonia were present.

*Animals Remaining Free of Secondary Respiratory Infection, 2nd Week to Termination of Experiment*

*A. Gross Appearance.*—Beginning with the 2nd week of exposure the gross appearance of the lungs became constant, and the pink and white mottling persisted. There was no consolidation, pleural effusion, or edema.

*B. Microscopic Changes.*—The hyperplasia and hypertrophy of alveolar cells continued and numerous mitotic figures were found throughout the experimental period. The alveolar walls were thickened by the increased alveolar cells, and there was a diminution in the number of blood-containing capillaries in the walls (Figs. 8 to 11). This increase in cellularity occurred in both young and old rats, and after the first 2 weeks of exposure no estimation of age could be made from the histological picture.

After 1 month of exposure the small arterioles of the lungs became prominent and apparently more numerous (Fig. 15). Their walls were thickened and the lumina narrowed. Later, hyalinization of the walls occurred and occasionally thrombosis (Figs. 13 and 15). These changes in the walls made the small vessels stand out prominently, which probably accounted for the apparent increase in number. Frequently vessels showed thrombosis which appeared to be due to a proliferation of the endothelial cells without any evidence of hyalinization. In vessels showing the most extensive involvement, the appearance was identical to that of the small renal arterioles undergoing hyalinization in chronic vascular nephritis. It is the type of lesion which is usually associated with hypertension. Measurements of the hearts of a representative group of rats failed to show any conclusive evidence of hypertrophy in either ventricle, although the right ventricle tended to be a fraction of a millimeter thicker in exposed animals in some instances. If true hypertension in the pulmonary circuit were responsible for these changes, one would have expected definite right-sided hypertrophy. It is fair to say, however, that this point was not adequately settled by studying these small animals.

A number of large pulmonary arteries began to show marked pathological changes about the 45th day of exposure. The walls of these vessels were thickened in an irregular manner. The media first assumed a loose meshed appearance with clear areas interspersed between the tissue elements. In places the media was hyalinized, and definite hyaline cartilage formation was frequently found (Fig. 14). In two instances calcification had occurred with these changes.

All rats autopsied during the last month of exposure showed the changes described in the above paragraphs.

*Removal to Normal Air after 72 Days Exposure*

On the 72nd day the first exposure was discontinued. The chamber was decompressed over a 2 hour period according to the stage method formulated by Boycott, Damant, and Haldane (6). The animals remained in the experimental chamber for the next 40 days

under normal atmospheric conditions. During decompression 28 per cent of the rats died, all with symptoms of acute asphyxia. These showed considerable fibrosis of the lungs, complicated frequently with bronchopneumonia.

The surviving animals appeared to be in excellent health during the interval in normal air and made rapid gains in weight (2). Autopsies were performed at frequent intervals. The cellularity of the alveolar walls and the vascular lesions continued to be present. Slight amounts of fibrosis and emphysema were seen, together with some increase in interstitial tissue. The lungs, on the whole, were in the same condition as they had been since the 2nd week of the first exposure.

*Reexposure to 83.6 Per Cent Oxygen Tension after 40 Days in Normal Air*

After 40 days in normal air, the remaining 35 rats were reexposed to the same conditions that existed during the first exposure. This second exposure lasted 10 days. During this time no changes occurred.

The important finding during this exposure was that none of the rats developed acute oxygen poisoning. They remained in excellent health, irrespective of the fact that all were now adults. On the 4th day, when the acute pulmonary edema was most intense during the first exposure, no alveolar exudate or congestion was found. Control rats developed classical oxygen poisoning beginning with the 3rd day of exposure.

On the 10th day of the second exposure the chamber was again decompressed and the animals returned to normal atmospheric conditions. Whereas 28 per cent of the survivors died during decompression after the first exposure, no deaths occurred this time, and there were no signs of temporary illness. The results of the second exposure may be summarized by saying that complete adaptation to the toxic action of an environment having an 83.6 per cent oxygen tension was produced by the first exposure, so that on reexposure no changes occurred either clinically or pathologically.

At the present writing, 214 days after conclusion of the experiment, all of the surviving animals are apparently in good health and are being retained for future study. 33 days after the end of the second

exposure three rats were autopsied. The lungs showed the same picture as during exposure, although the hypertrophy of alveolar cells was somewhat less marked than formerly.

#### DISCUSSION

An explanation for the adaptation of rats to toxic oxygen tensions probably involves both physiological and morphological changes. The alveolar walls of young rats are much more cellular and thicker than those of normal old rats. Young rats do not develop the edema of acute oxygen poisoning, whereas old rats show the classical pulmonary edema. After prolonged exposure to a toxic oxygen tension the alveolar cells of both young and old rats become hyperplastic and hypertrophied, causing a great increase in the thickness of the alveolar walls, and the two can no longer be distinguished histologically. These changes persist even after removal to normal oxygen tensions. On reexposure to toxic tensions no signs of oxygen poisoning are found in either the young or old. In other words, the lungs of old rats were "made young" during the first exposure, so that on reexposure old rats behaved as young ones did on first exposure.

Although the morphological difference between the lungs of rats under 3 months of age and those over 6 months old can be correlated with the increased resistance of young rats and the adaptation of old rats to 83.6 per cent oxygen tensions, this cannot fully explain the differences between the reaction of young, old, and adapted rats. No obvious difference between the morphology of the lungs of 1 month old rats and 3 months old rats can be detected; nevertheless, a definite increase in susceptibility occurs gradually with advancing age, as manifested by the increasing severity of the clinical symptoms. Therefore, this change in resistance with advancing age can only be explained by some physiological alteration in the body. The fact that marked histological changes are produced in the lungs of rats by prolonged exposure indicates that the defensive mechanism responsible for this increased resistance is at least in part in the lungs. We believe that an alteration in the physiology of the alveolar cells occurs, and that this is the primary factor in the adaptation which results, and in the difference in the susceptibility of various age groups.

Our findings permit three possible explanations for the higher re-

sistance of young or of adapted older animals. First, the increase in the thickness of the alveolar walls may mechanically decrease the rate of diffusion of the alveolar gases, so that at increased oxygen tensions the total amount of oxygen reaching the capillaries is only sufficient to approximate the oxygen tension of the blood under normal atmospheric conditions.

Second, the fact that definite vascular changes have been demonstrated after prolonged exposure points toward the possibility that generalized alterations in the physiological structure of the capillary bed in the lungs may be responsible for this adaptation.

Third, the marked increase in number and size, as well as the changed appearance of the alveolar cells, forces one to consider the possibility of some change in their physiological properties. Although the available evidence has been accepted by the majority of physiologists to mean that the gaseous pulmonary exchange is a process of simple diffusion, the results of these experiments compel one to consider the possibility of the alveolar cells engaging in a process of physiological activity under certain environmental conditions. The strongest evidence for this theory is the fact that adapted animals remained in perfect health and showed no clinical symptoms of dyspnea or anoxemia on sudden removal to normal oxygen tensions. If the mechanical theory of impaired diffusion were the sole factor, one would expect to find definite signs of respiratory distress at least temporarily after removal to lowered oxygen tensions. On the other hand, this does not eliminate the rôle that might be played by an increase in the resistance of the capillary endothelium.

#### CONCLUSIONS

1. The alveolar cells in the lungs of young rats are greater in number and size than in old rats, a gradual transition to the state in the latter occurring from about the 4th to the 6th months of life.

2. On prolonged exposure to an environment having an 83.6 per cent oxygen tension, the cellularity of the alveoli is increased in both young and old animals, so that after 2 to 3 weeks it is impossible to distinguish them by morphological differences. Numerous mitotic figures are present in the alveolar cells. This hyperplasia and hypertrophy is a change which persists for months after the rats return to normal air.

3. Young rats do not develop the symptoms of acute oxygen poisoning, although some perivascular edema and dilatation of the lymphatics results on the 4th day of exposure, when intense acute pulmonary edema is present in old rats.

4. The mortality of acute oxygen poisoning is directly proportional to the age of the animals, although the majority of rats under 6 months of age survive this state and continue in apparent good health for as long as 72 days. All deaths during the acute stage in an 83.6 per cent oxygen tension occur on the 4th day of exposure.

5. After 1 month of exposure lesions are to be seen in the small arterioles of the lungs, consisting of a thickening and hyalinization of the walls with ultimate thrombosis of many. These vascular changes are identical with those seen in the arterioles of the kidney in chronic vascular nephritis.

6. Around the 45th day of exposure the large pulmonary arteries contain lesions in the media. The walls become loose meshed, thickened, and hyalinized, and hyaline cartilage formation is associated with these changes.

7. Reexposure of animals following an interval of 40 days in normal air subsequent to the first exposure of 72 days, does not produce any clinical or pathological changes. An adaptation to this toxic oxygen tension is produced during the first exposure, so that oxygen poisoning does not occur on second exposure. The increased cellularity of the alveolar walls persists.

8. The similarity in the morphological structure of the alveoli in young rats and in previously exposed old rats has a definite relationship to the adaptation that occurs to an oxygen tension of 83.6 per cent, preventing the development of acute oxygen poisoning on re-exposure.

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## EXPLANATION OF PLATES

The stain used for all the microscopic preparations was hematoxylin and eosin.

## PLATE 2

FIG. 1. This field shows the architecture of the lung of an adolescent rat. The cellularity and thickness of the alveolar walls are well represented. Normal, young rat, 48 days old, male. Camera lucida drawing.  $\times 80$ .

FIG. 2. The histology of the normal adult rat lung is illustrated in this drawing. The characteristic thinness and diminished cellularity of the alveolar walls are easily seen. Several small blood vessels are present showing the normal relation of the perivascular tissues. A comparison of this drawing with Fig. 1 shows the relative difference in the thickness and cellularity of the alveolar walls in young and adult rats normally. Normal rat, 277 to 288 days old, male. Camera lucida drawing.  $\times 80$ .

FIG. 3. Magnification of the field outlined in Fig. 1. The cellularity and thickness of the alveolar walls in normal young rats is seen more clearly. The relatively large size of the alveolar cells is also well represented. Camera lucida drawing.  $\times 340$ .

FIG. 4. Magnification of the field outlined in Fig. 2. A comparison of this drawing with Fig. 3 shows the differences in the detailed histology of the lungs of normal old and young rats. The alveolar cells are more numerous and larger in young rats than in old, causing the alveolar walls to be thicker in the former. Camera lucida drawing.  $\times 340$ .

## PLATE 3

FIG. 5. Zones of perivascular edema are prominent, and these are the only changes found in young rats on the day when acute oxygen poisoning is most intense in old animals. The alveoli contain no exudate. Female rat, 37 days old. Removed from 83.6 per cent oxygen tension in excellent health after 4 days of continuous exposure, and killed for autopsy. Camera lucida drawing.  $\times 155$ .

FIG. 6. This area shows an extensive alveolar exudate consisting of fibrin, desquamated alveolar cells, and polymorphonuclear leucocytes. The capillaries of the alveolar walls are studded with polymorphonuclear leucocytes. Female rat, 437 to 444 days old. Removed from 83.6 per cent oxygen tension when acutely ill after 4 days of continuous exposure, but died during the decompression to normal oxygen tension. Camera lucida drawing.  $\times 735$ .

FIG. 7. This shows well the diffuse serous exudate in the alveoli, with some fibrin and cellular debris. Also, one sees the striking zones of perivascular edema and the dilated lymphatics. Male rat, 122 to 130 days old. Found dead after 4 days of continuous exposure to 83.6 per cent oxygen tension. Camera lucida drawing.  $\times 145$ .

## PLATE 4

FIG. 8. This slide illustrates the increased cellularity and thickness of the alveolar walls which occur after prolonged exposure. Also, one sees small, patchy areas of atelectasis interspersed between expanded alveoli. Note that the alveoli contain no exudate, and there is no perivascular edema. Female rat, 87 days old. Removed from 83.6 per cent oxygen tension in good health after 49 days of continuous exposure, and killed for autopsy. Camera lucida drawing.  $\times 80$ .

FIG. 9. A comparison of this slide with Fig. 8 shows the similarity of the histological structure of the lungs of old and young rats after prolonged exposure. They can no longer be differentiated by morphological differences. Contrasting this drawing with Fig. 2, the increase in the cellularity of the alveolar walls after exposure is readily seen. Patchy areas of atelectasis are present. The alveoli contain no exudate. Male rat, 170 to 180 days old. Removed from 83 per cent oxygen tension in good health after 44 days of continuous exposure, and killed for autopsy. Camera lucida drawing.  $\times 80$ .

FIG. 10. Magnification of the field outlined in Fig. 8. This drawing is to be compared with Fig. 3 in order to emphasize the increased cellularity of the lungs of young rats after prolonged exposure. The increase in the size of the alveolar cells and the nuclei is also clearly seen. Camera lucida drawing.  $\times 340$ .

FIG. 11. Magnification of the field outlined in Fig. 9. A comparison of this drawing with Fig. 4 shows the marked increase in cellularity of the lungs of adult rats after prolonged exposure. A comparison with Fig. 10 shows the morphological similarity of the lungs of old and young rats after exposure, in contrast to the difference in the structure of the lungs of normal old and young rats. Camera lucida drawing.  $\times 340$ .

## PLATE 5

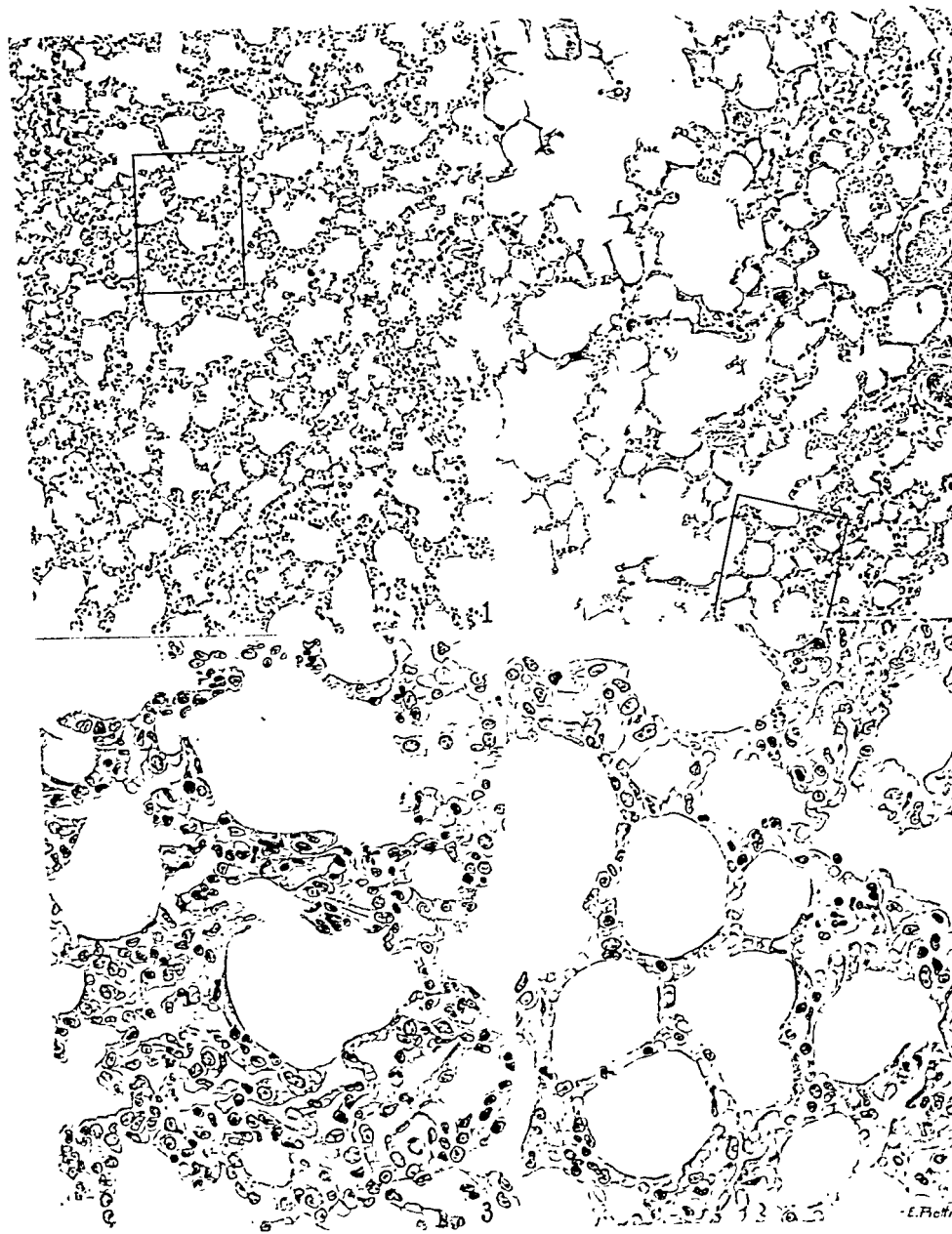
FIG. 12. Mitotic figure in an enlarged alveolar cell from Fig. 9. Camera lucida drawing.  $\times 1260$ .

FIG. 13. Thickened, hyalinized wall of a small pulmonary arteriole, with endothelial proliferation. Male rat, 178 to 188 days old. Found dead in 83.6 per cent oxygen tension after 52 days continuous exposure. Camera lucida drawing.  $\times 390$ .

FIG. 14. A large branch of the pulmonary artery is shown, illustrating the thickening of the media with hyaline cartilage formation. Female rat, 217 to 220 days old. Exposure was 30 days in 83.6 per cent oxygen tension, 53 days in normal air, than 44 days reexposure to 83.6 per cent oxygen tension. Camera lucida drawing.  $\times 115$ .

FIG. 15. The same slide as Fig. 13. Five small blood vessels stand out prominently because of the hyalinization and thickening of the walls. Endothelial proliferation with thrombosis is seen in one. The alveolar epithelial cells show some desquamation due to postmortem changes. Camera lucida drawing.  $\times 155$ .





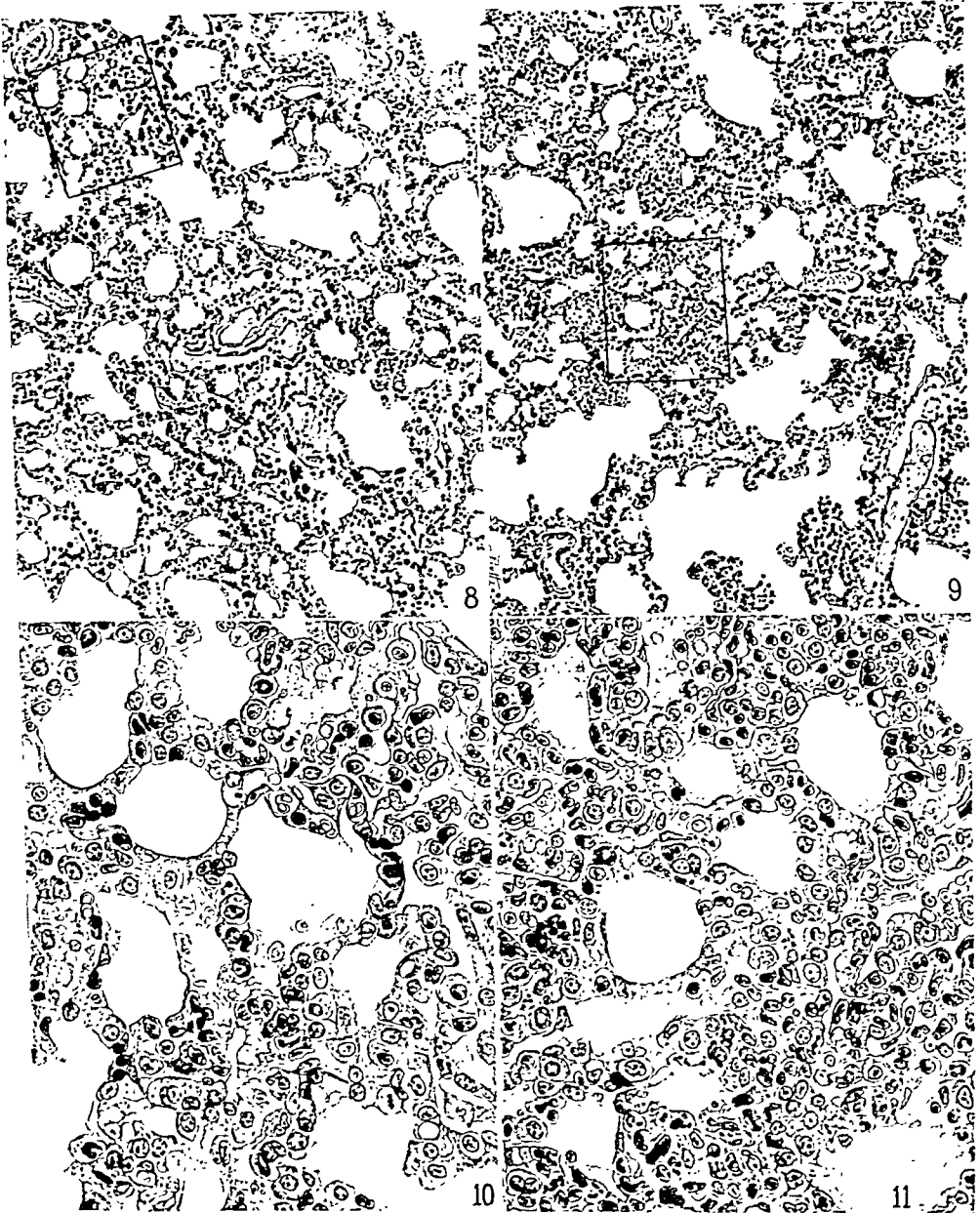
(Smith *et al.*: Lung changes under compression)





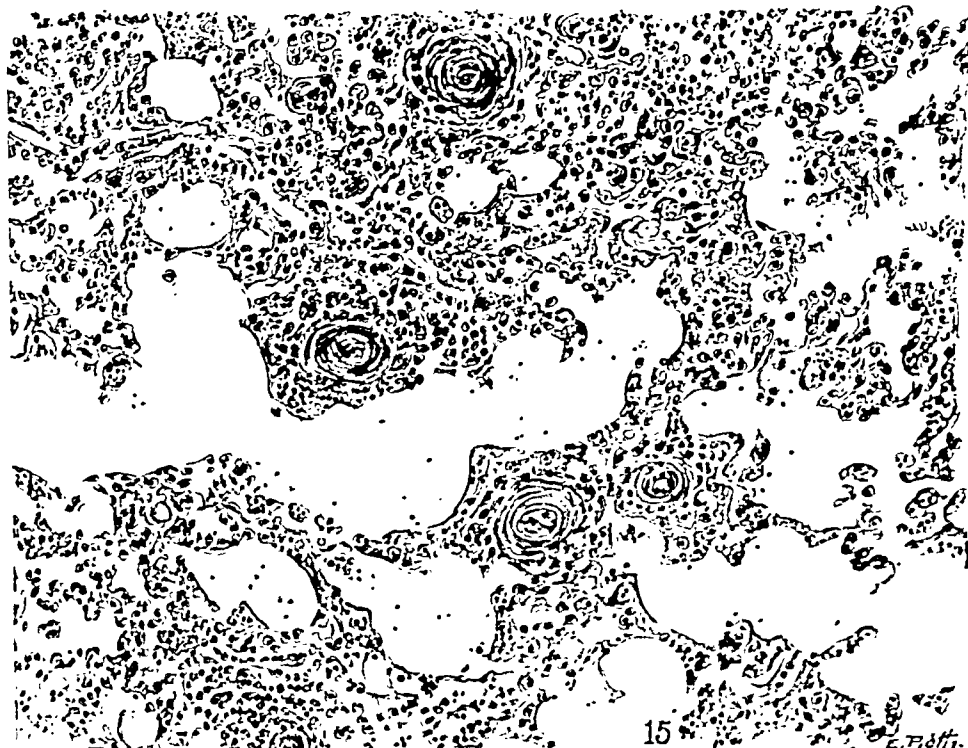
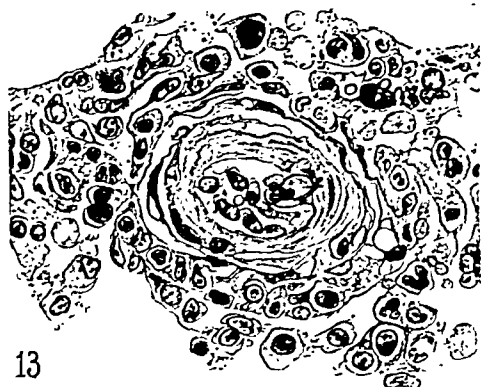
(Smith *et al.*: Lung changes under compressed air)



(Smith *et al.*: Lung changes under compressed air)







E. Rott.

by its agent, the limitation to special breeds of fowls in the early transfers, and the fact that the agents cause specific cells to differentiate into definite types of tissue suggest that the process represents a perverse physiological phenomenon. On the basis of this conception it has seemed justifiable to set up as a working hypothesis the possibility that the tumor agents are of endogenous origin, representing abnormal activities of the forces which normally control growth and differentiation of tissues. A series of investigations to test this idea have been carried out, in which the properties of the tumor agents have been contrasted with those of typical viruses on the one hand, and with cell products such as enzymes on the other.

Two studies of the series have been published. The first (1) reported the fact that the tumor agent is fixed or inactivated *in vitro* by the mesodermal tissues of susceptible fowls, but not by the epithelial tissues, while none of the tissues of non-susceptible animals affect the agent. In this respect the agent resembles many cell products which are fixed by the specific substrate on which they act. As a contrast to this finding, the viruses were not fixed *in vitro* by tissues most susceptible to infection, but had their infectivity greatly increased by the contact (2). The second investigation (3) was on the quantitative and qualitative action of ultraviolet light on the tumor agent. Bacteria, viruses and phage have been shown to be injured in much the same degree by given wave lengths in this general range of the spectrum. On the other hand, the chicken tumor agent is not only far more resistant to these wave lengths, but there is a striking qualitative difference in that the most active wave lengths for the agent do not correspond to those for the other group. This result is interpreted as indicating a common factor in the bacteria-virus-phage group and it suggests that inactivation of the tumor agent is due to a destruction of a substance having an entirely different spectrum from the group mentioned and therefore of a different chemical character.

The present study is an attempt to gain further information on the nature and properties of the chicken tumor agents by the relatively direct method of isolation and purification. This will be followed by a publication on the antigenic properties (4) of the agent and another presenting the evidence of an inhibiting factor associated with the agent (5).

#### *Fractionation of Active Tumor Filtrates*

As a first step in this investigation we have fractionated active tumor extracts in order to determine whether the activity is associated

with one of the recognized proteins, as is the case with most if not all of the effective cell products.<sup>2</sup> Three means have been used in attempts to obtain different fractions from the extracts; namely, reduction of the salt content and the increase of hydrogen ion concentration by direct addition of acid or with proper buffered solutions.

*Electrodialysis.*—About 30 gm. of fresh tumor tissue were ground and thoroughly extracted with 600 cc. of water and filtered through a Berkefeld candle. This filtrate was rapidly concentrated to about 1/10 its original volume in thimbles lined with an 8 per cent collodion membrane. The concentrate was subjected to electrodialysis according to the Bronfenbrenner (6) method. The precipitate

TABLE I

Experiment No.	Material	Time of dialyzing min.	Precipitate		Fluid from dialysate		Control positive	pH of dialysate
			No. of inoculations	Positive	No. of inoculations	Positive		
1	Berkefeld filtrate	7						
2	" "	5	4	100	4	0	100	4.5
3	" "	3	4	50	4	0	50	4.3
4	" "	5	4	50	4	0	100	
5	" "	5	4	0	4	0	100	
6	" "	5	4	0	4	0	100	
7	H <sub>2</sub> O extract dry	5	6	100	4	0	100	4.5
8	tumor serum	5	12	91.7	6	0	100	4.4
	Concentrated tumor chicken	8	6	66.6	12	0	100	4.7
		3		66.6	6	0	50	
				3	0			

which occurred was clumped and usually adhered to the positive pole, and could easily be separated from the fluid. It was then washed, dissolved and injected intradermally into chickens. The fluid was neutralized and also injected.

In the first group of experiments the electrodialysis was continued until an amperage corresponding to that of distilled water was reached, the time required varying from 45 minutes to 2 hours. The heavy precipitate secured and the fluid were both inactive. In a new group of experiments the time of electrodialysis was shortened to 15 minutes,

<sup>2</sup> Sugiura and Benedict (*J. Cancer Research*, 1927, 11, 164) have reported that the tumor agent can be salted out from a filtrate with the globulin fraction.

of analyses is given in Table II. On hydrolysis a reducing substance was found present in all the precipitates, representing about 15.25 per cent figured as glucose. The ratio of nitrogen in precipitates to that of intact extracts varies with the method of preparation of the extract. With the concentrated Berkefeld filtrate from 80 to 90 per cent of the nitrogen goes into the precipitate, while with extracts of tumor desiccate, which have a higher nitrogen content, the amount carried by the precipitate may be as low as 60 per cent of the total amount. If the mixtures are kept slightly alkaline during extraction, the percentage of phosphorus is increased. There is little change in the physical properties or chemical constituents of the fraction repeatedly dissolved in alkali and reprecipitated with lactic acid. It was considered that the fraction was either a mixture of proteins or a protein of unusual constitution. Study of this point was rendered unnecessary by the results of the following experiments.

TABLE II

Method of precipitation	N	P
	<i>per cent</i>	<i>per cent</i>
Electrodialysis.....	12.47	0.27
Lactic acid.....	12.64	0.29
Sodium acetate buffer.....	13.33	0.22

*Separation of the Tumor Agent from the Bulk of the Proteins*

As further attempts to isolate and purify the protein associated with the tumor agent failed because the necessary procedure inactivated the material, other methods of accomplishing the purpose were sought. We had previously undertaken to adsorb the active principle from the filtrate on aluminum hydroxide and then to release it by treatment with an alkaline fluid, but at the time the results were considered too irregular to justify an extension of the work along this line. Other investigations have shown that a variety of substances adsorb or inactivate the tumor agent (7). Leitch has shown that the active material may be released after adsorption on kaolin and more recently Fränkel has reported some success in releasing it after adsorption on aluminum hydroxide (8), but his results were irregular

in that the released agent was not highly active and sometimes failed to induce tumors. It was considered worth while to reexamine the possibility of utilizing this method for our purpose.

*Method.*—For the tumor agent we used either a Berkefeld filtrate of a fresh extract concentrated in a collodion membrane or an extract of tumor desiccate. The solutions were kept at a pH of about 7.2 during the process of preparation by the addition of  $x/100$  NaOH. Type C aluminum hydroxide was prepared according to the method described by Willstätter and Kraut (9). 20 cc. of the tumor extract were mixed with 20 cc. of the aluminum hydroxide suspension. After thoroughly shaking, the mixture was centrifuged and the supernatant fluid, which for convenience will be referred to as the aluminum supernatant, was decanted. The deposit was washed several times with distilled water, and the washing concentrated to 20 cc. in an 8 per cent collodion membrane. Enough of the washed aluminum deposit was set aside for inoculation and the remainder was shaken for 5 minutes with 20 cc. of  $x/15$   $\text{Na}_2\text{HPO}_4$  at a pH of 8, centrifuged and the supernatant fluid drawn off. This will be referred to as the released material.

For testing the activity of the various products, they were inoculated intradermally in chickens, every fowl receiving 0.2 cc. of each test material. These included the following: (a) original tumor extract, (b) the supernatant fluid after the aluminum hydroxide with its adsorbed material had been separated from the extract, (c) concentrated washing, (d) the aluminum hydroxide after washing and (e) the material released from the aluminum by shaking with  $\text{Na}_2\text{HPO}_4$ .

In later experiments the technique was modified in one particular to avoid unnecessary dilution of the material. The 20 cc. of the aluminum hydroxide were first centrifuged and the excess fluid discarded before the addition of the tumor extract.

A large number of experiments was carried out by these methods, the results of 9 of which are given in Text-fig. 1.<sup>3</sup> In addition a great many more, in which the animal inoculated was to test the activity of the released material for chemical study, yielded similar results.

<sup>3</sup> By using the intradermal inoculations it is not only possible to secure more accurate measurements, but by having each fowl receive control and several test materials a better comparison of the growth rate of the induced tumors can be arrived at. The period of observation was from 3 to 5 weeks. To eliminate the variations due to the difference in potency of the extracts and the susceptibilities of the individual chickens we selected the measurement taken at the time when the control tumors had reached a certain size. The more susceptible fowls with a very active extract may reach this point in 2 weeks, while the more resistant ones will require 4 weeks. This system has been utilized in arriving at the figures given in all of the charts.

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From the results shown in Text-fig. 1 it is evident that a certain amount of the active material is either directly adsorbed on the aluminum or is carried down along with some adsorbed substance, as Fränkel has reported. That the amount of the agent carried down with the aluminum hydroxide represents only a fraction of the total amount present in the tumor extract is shown by the results of inoculations, for not only is the percentage of tumors induced by the released substance low, but the tumors obtained are of relatively small size. The increased potency of the agent remaining in the supernatant fluid of the tumor extract after removal of the aluminum hydroxide is unquestionably due to the elimination of an inhibiting substance, as

Release of chicken tumor agent  
after adsorption on aluminum hydroxide  
(9 experiments)

Material inoculated	No. of inoculations	Per cent tumors	No. of tumors	Average size of tumors
Tumor extract	20	100	20	 1.4 × 1.2 cm
Eluate	41	60.9	25	 1.3 × 1.0 "
Supernatant fluid	20	100	20	 2.3 × 1.8 "

TEXT-FIG. 1. All inoculations were made intradermally, distributed so that each fowl received at least 1 injection of each test material. The size of the tumors in each individual was recorded when that arising from the control inoculation had reached about the size indicated in the above figure.





will be shown in another paper. However, allowing for this factor, the activity is such as to indicate that the concentration of the agent in this supernatant fluid has been little reduced by the aluminum adsorption.

#### *Effect on the Tumor Agent of Variation of the Quantity and Type of Adsorbent*

The selection of adsorbent and the ratio of adsorbent to extract as used in the foregoing experiments was more or less arbitrary. Improvements in method were sought by additional experiments in which the ratios used above were varied, and also Willstätter's three other types of aluminum hydroxide were tested.

*Variation in Ratio of Adsorbent to Extract.*—Tumor extracts prepared as described above were mixed with aluminum hydroxide Type C in the following proportions: 1:3, 1:2, 1:1, 1:1/2, 1:1/4 and 1:1/10. The mixtures were thoroughly shaken, centrifuged and the supernatant fluids injected intradermally in chickens. The untreated extract was also injected into each chicken for control. The results of these experiments with the proportions of 1:1 to 1:1/10 are shown in Text-fig. 2. 5 such experiments were carried out, in which 19 test inoculations were made of each test ratio. There were a few tests with higher ratios of aluminum hydroxide, in which the tumors produced by the supernatant fluid were considerably smaller.

On the whole the results given in Text-fig. 2 are of relative importance only, the tests being necessarily crude, since there is considerable variation in the activity of the extracts and the adsorbing power of

Activity of tumor extract after adsorption with varying proportions of aluminum hydroxide				
Ratio of tumor extract to aluminum hydroxide	1:1	2:1	4:1	Control
Number of inoculations	19	19	19	19
Average size of tumors (cm.)	2.07 × 1.46	1.73 × 1.35	1.67 × 1.20	1.60 × 1.20
				

TEXT-FIG. 2. The above results are based on the average size of tumors induced in 5 experiments in which all inoculations resulted in tumors. Each chicken received an intradermal inoculation of each of the test materials and the measurements recorded in the figure were based on those of all tumors in an individual at the time when the tumor from control inoculations had reached about the size indicated.

the aluminum hydroxide varies with time. However, even allowing for the two variable factors, the supernatant fluid of the 1:1 mixture was plainly the most active in tumor production in practically every experiment.

*Action of Different Types of Aluminum Hydroxide.*—Comparative tests have been made with Willstätter's four types of aluminum hydroxide.

The technical procedure was essentially the same as that described above. Mixtures were made of chicken tumor extract and of Types A, B, C and D aluminum hydroxide in a ratio of 1:1. The mixtures were shaken and centrifuged and the supernatant fluid injected intradermally in chickens. The injections were so

distributed that each of the chickens received an inoculation of the supernatant fluid from the four types of aluminum and a control inoculation of the original tumor extract. The tumors produced by the supernatant fluid from Types B and D aluminum hydroxide were little if any larger than the controls, while that from Type A was smaller. The results with the supernatant fluid from Type C in this experiment were similar to those reported in the previous experiments, in that the tumors were considerably larger than those produced by the original tumor extract.

While this experiment also is crude, owing to the fact that the amount of aluminum hydroxide by weight is not accurately indicated by volume of the different preparations, from the point of view of our objective, the separation of the tumor agent from contaminating material, the results indicate that Type C aluminum hydroxide is the most satisfactory preparation for this purpose. In subsequent studies this type was used exclusively.

#### *Nature of the Tumor Extract after Adsorption with Type C Aluminum Hydroxide*

The aluminum supernatant fluid is derived from either a concentrated Berkefeld filtrate from fresh Chicken Tumor I material or from a water extract of a desiccate of the tumor. When the filtrate of fresh tumor is used, the supernatant after removal of the aluminum hydroxide is a clear, colorless fluid with a high viscosity. If an extract of tumor desiccate is the source, the fluid is equally viscous and is generally opalescent, a property probably due to lipoids. A detailed chemical study of this material will be published later, but a summary of the preliminary work follows.

*Nitrogen Content.*—The nitrogen content of the concentrated filtrates and extracts of dry material shows considerable variation, but on the average of some 12 analyses is 0.527 mg. per cc. for filtrates and for the extracts of desiccates is 0.724. Of this amount 92.60 per cent is adsorbed on the aluminum hydroxide from the filtrates and 86.47 per cent from the extracts of dry tumor. Of the amount adsorbed on the aluminum about 27 per cent is found in the released material.

*Reducing Substances.*—On hydrolysis of the aluminum supernatant fluid a reducing substance is found, which, figured as glucose, amounts to 0.175 mg. per cc. This represents about 1/3 of the amount present in the full tumor extract, indicating that this substance is adsorbed in a smaller ratio than the nitrogen-containing

substances. The ratio of nitrogen to sugar in the full extract is 1 to 0.95, while in the aluminum supernatant fluid it is 1 to 3.48.

While the usual protein-precipitating agents, such as acetic, tannic, tungstic and trichloroacetic acids, produce no precipitate in the aluminum supernatant, salts of the heavy metals such as lead, silver and mercury, and the basic dyes, safranin and neutral red, do give precipitates. The biuret, Millon, Adamkiewicz's and xanthoproteic tests are negative. Molisch and Tollens tests are positive.

The biological tests for protein have been negative. 9 guinea pigs, injected intraperitoneally with 8 cc. each of intact tumor extract, showed no anaphylactic symptoms when given 14 days later from 2 to 10 cc. of aluminum supernatant fluid intravenously. Furthermore no sensitization was induced in 14 animals by the injection of even the equivalent of 40 cc. of highly active aluminum supernatant, as demonstrated by the absence of anaphylactic symptoms when a second injection of 2 to 10 cc. of the same material was given 12 days later. However, there is some sensitization induced in these animals to an intravenous injection of unpurified tumor extract. As a further indication of the very low protein content of the aluminum supernatant, the sera of rabbits repeatedly injected with the material, while showing neutralizing antibodies, gave no evidence of complement-fixing antibodies.

#### *Removal of Viscous Material from Aluminum Supernatant Fluid*




The properties of the aluminum supernatant fluid as outlined above indicate that the protein content is extremely small, and that the main constituent is probably a carbohydrate. The fact that the tumor extracts contain muco-protein, and that the aluminum supernatant fluid contains a viscous substance which behaves like an acid, suggested that the latter has properties similar to chondroitin-sulfuric acid. In attempts to eliminate it as a further step in purification, the direct removal by precipitation with such agents as the salts of heavy metals or basic dyes was found to destroy the tumor-producing activity of the solution. One of us (Claude) conceived the idea that the removal might be accomplished by combining the substance with a basic protein. Gelatin was selected for the reason that it has no antigenic properties and is not precipitated by acids under the conditions of the experiment.

*Experiment.*—It was found by preliminary tests that when gelatin is added to aluminum supernatant fluid and the pH of the mixture brought to between 4 and 4.8 with 1/10 acetate buffer a precipitation is induced. No precipitation occurs in either the aluminum supernatant fluid or the gelatin solution alone when the acid is added. On the basis of these findings the following method was evolved

for use in the experiments: to 10 cc. of aluminum supernatant fluid of a chicken tumor extract, prepared as described above, was added 1 cc. of a 2 per cent solution of commercial gelatin (Gold Label). Sufficient M/10 acetate buffer at pH 4.7 was added to bring the solution to pH 4.8. After 10 minutes the fluid was centrifuged, the supernatant fluid filtered through filter paper, the precipitate washed with acetate buffer at pH 4.7 and dissolved in a sufficient amount of Ringer's solution to bring the volume up to that of the supernatant fluid.

The gelatin supernatant fluid proved to be water-clear and limpid. No precipitate was formed by neutral red or basic lead acetate. The presence of excess gelatin did not permit of any conclusion from the nitrogen and sugar determinations. The dissolved gelatin precipitate, when treated with neutral red or lead acetate, gave a precipitate of the same character as that derived from the aluminum supernatant fluid and a substance could be extracted having all the physical

Effect of removal of viscous material  
from aluminum supernatant fluid of tumor extract  
(21 experiments)

Material inoculated	No. of inoculations	No. of tumors	Average size of tumors
Aluminum supernatant fluid	36	33	 2.2 × 1.8 cm.
Gelatin supernatant fluid	45	29	 2.4 × 1.9 "
Gelatin precipitate	19	10	 1.7 × 1.6 "

TEXT-FIG. 3. The same system of recording the sizes of tumors was used here as in the preceding text-figures.

properties of the viscous fluid referred to above. Guinea pigs sensitized by the injection of 8 cc. of full strength chicken tumor filtrate, aluminum supernatant fluid or gelatin supernatant fluid show no anaphylactic symptoms when given gelatin supernatant fluid intravenously. This statement is based on the results on 18 animals tested. There are symptoms however in animals sensitized with the gelatin supernatant fluid and subsequently injected with unpurified tumor extract. This result is similar to that with the aluminum supernatant fluid recorded in the preceding section.

The tumor-producing activity of the gelatin supernatant fluid and the dissolved precipitate was tested by intradermal injection of 0.2 cc. of each into chickens which also received an equal amount of the aluminum supernatant fluid from which these products had been derived. The results of 21 experiments in which the relative activity of these three materials was tested by inoculation of 36 chickens are shown in Text-fig. 3.

The results of these investigations show the possibility of eliminating one more impurity from the tumor extract without interfering with the activity. There is some difficulty in judging the comparative tumor-producing activity of the aluminum supernatant fluid and the gelatin supernatant fluid, because gelatin enhances the tumor-producing property of the agent.<sup>4</sup> The fact that an appropriate amount of the latter is adsorbed on the gelatin precipitate, and yet the amount left in the supernatant fluid is still capable of inducing tumors as large as or larger than those resulting from the injection of aluminum supernatant fluid, renders it very improbable that the viscous substance is involved in tumor production. Unfortunately the presence of protein cannot be determined on account of our inability to eliminate the excess gelatin; but the failure of this material to sensitize guinea pigs to a subsequent injection, and the fact that pigs sensitized to an unpurified tumor extract do not react to an injection of gelatin supernatant fluid indicates that an infinitesimal amount of protein is present or that the protein is non-antigenic. It is hoped that other methods can be developed for eliminating the viscous material which do not introduce factors interfering with direct chemical analysis.

#### DISCUSSION

The principal basis on which the chicken tumor agents are considered to be viruses is that they are deemed to be capable of self-perpetuation. It is established beyond doubt that these agents are definitely increased in amount with the propagation of the tumor. However, with increasing knowledge of their properties, certain apparently fundamental differences between this group and the animal viruses make it seem unlikely that they belong to the same order. Some of these have already been discussed. Such facts as the sharp difference in susceptibility to ultraviolet light, both quantitative and qualitative, alone suggested a wide gap between the tumor agents on one side and bacteria, viruses and phage on the other. The affinity between the tumor agent and susceptible tissues *in vitro* has no parallel among the parasites. We now have evidence for the association of the agent with a protein fraction, and for its possible dissociation from

<sup>4</sup> Unpublished observation by Murphy and Sturm, confirmed and extended by Claude.

the protein. In subsequent papers the presence of an inhibitor principle in tumor extracts and its peculiar antigenic properties will be brought out (10). Certain animal viruses can be carried down from a suspension with a precipitated protein (11), but so far none has withstood the wide range of pH variation and the vigorous chemical handling incident to repeated distribution in fluid and reprecipitation tolerated by the tumor agent. Viruses may be infective in dilutions so great that chemical tests fail to indicate the presence of protein, but the reduction of protein in a virus suspension is accompanied by evidence of reduction of the infective units present (12). On the other hand, tumor extracts cannot be diluted very much and still retain sufficient concentration to induce tumors (13).

In discussing a possible classification other than among the viruses we have previously used the term enzyme-like (14), a term meant to indicate the possible production of the active material by tissue cells. No closer analogy to enzymes is considered, for most of the evidence suggests that the tumor agents belong to a class not yet clearly defined. That a product of an abnormal cell can cause a normal cell of the same derivation to develop into an abnormal cell of the same type from which the product came, and in its new form be capable of producing more of the active material, has seemed to many a fantastic conception. At the time that this hypothesis was brought forward, there was no clear-cut example of such a phenomenon. Now it is known that a substance may be extracted from a type-specific pneumococcus which will cause avirulent, non-specific pneumococci to change to the virulent form of the same type from which the extract was obtained (15). In its new form the organism produces more of the active material and transmits the property to its descendants. If we can call this active substance an agent, and three distinct active chemical substances have been obtained from the pneumococci, there is undoubtedly evidence that such agents increase, or more precisely, are increased with the cultivation of the organism. Yet these agents are products of the virulent cells and are not viruses. The effect of these agents may properly be referred to as a mutation. As there are other perhaps less well known examples of this phenomenon among bacteria (16) lacking proper designation, the term transmissible "mutagen" has been suggested for the group (17). With present

knowledge it seems probable that the chicken tumor agents have closer analogy to the mutagens than to the viruses.

## SUMMARY

By two methods a protein fraction can be separated out from a Chicken Tumor I extract, which carries all the tumor-producing agent. The precipitate can be dissolved and reprecipitated a number of times without loss of activity. The agent can be largely dissociated from the protein as shown by the fact that aluminum hydroxide will adsorb the protein from an extract and leave the agent behind. This purified material has a very low protein content, if any, as shown by both chemical and biological tests.

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# PROPERTIES OF THE CAUSATIVE AGENT OF A CHICKEN TUMOR

## IV. ASSOCIATION OF AN INHIBITOR WITH THE ACTIVE PRINCIPLE\*

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In earlier papers the nature of the chicken tumors has been discussed and some doubt expressed as to the basis on which the etiologic agents are considered to be filterable viruses (1). The idea has been advanced that these active principles may possibly be of endogenous origin, representing abnormal manifestations of the forces which normally control growth and differentiation of cells. Accepting this point of view as a working hypothesis, a series of tests has been devised with the expectation that the hypothesis may be either established or discarded. The present paper is a report of the evidence we have thus far obtained that indicates the presence of an inhibitor and represents an amplification of a previous publication<sup>1</sup> (2). Sittenfield, Johnson and Jobling have also published some evidence of the presence of such a factor in chicken tumors (3).

In an extensive series of experiments in which an attempt was made to isolate the tumor agent, it was noted that it could be precipitated out from the tumor extract along with certain of the proteins (4). Among other tests applied to the active precipitate was the Feulgen microchemical staining reaction for nucleoprotein. As a parallel to this, the precipitates were also tested with Mallory phosphotungstic stain, which differentiates intercellular material, cell protoplasm and nucleus. A correlation between the staining reactions of the precipitate and the tumor-producing activity of the extract showed that the more active material gave a strong Feulgen test and a clear yellow-red color with the Mallory. With less active material the Feulgen reaction was not so pronounced, and the Mallory gave a deep maroon-red. Finally with filtrates or extracts of dry tumor, having a

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\* This investigation was carried out under the Rutherford Donation.

<sup>1</sup> While this article was in press the paper by Sittenfield, Johnson and Jobling appeared.

very low grade of activity, the Feulgen reaction was faintly positive, while the Mallory showed blue-staining material predominating in the precipitate. These empirical observations, which seemed to indicate that the more active extracts contained a higher ratio of nuclear material, together with the fact that an extract of desiccated chicken tumor often has a low tumor-producing power while the residue is quite active, led to the following experiment.

### *Serial Extraction of Dry Tumor*

On the assumption that the blue-staining material found in the Mallory test might be in some way responsible for the low grade activity of the tumor extract in which it is most abundant, we have attempted to eliminate it.

*Experiment.*—1 gm. of a finely powdered tumor desiccate was extracted with 60 cc. of distilled water, by first rubbing the desiccate into a smooth paste in a mortar and then thoroughly mixing it by drawing it back and forth in a syringe. The mixture was centrifuged and the supernatant fluid filtered through filter paper. Chickens were inoculated intradermally with 0.2 cc. of the extract and with 0.1 cc. of the residue. The remaining residue was extracted again with 60 cc. of water, thoroughly mixed by pumping back and forth in a syringe and then centrifuged. The supernatant fluid was passed through filter paper and 0.2 cc. of this second extract and 0.1 cc. of the residue injected intradermally. This procedure was repeated eight times and each extract and each residue tested for its activity. The results in tumor production are shown in Text-fig. 1, which represents the average of 7 experiments, and in Text-fig. 2, giving one of several tests in which the first extract was inactive.

The nitrogen content of the extracts, indicating the amount of protein present, is shown in Text-fig. 3, which also shows the phosphorus content and the amount of reducing substance, figured as glucose. These figures are based on the average from 3 experiments. Over 60 per cent of the soluble protein, as indicated by the nitrogen present, is found in the first extract, while the third extract—which is the most active in tumor production—has only about  $1/4$  as much. The fourth extract, which is almost as active as the third and far more active than the first, has only 0.08 mg. of nitrogen per cc. The reducing substance decreased at almost the same ratio. These figures are based on analyses carried out by Dr. O. M. Helmer.

As will be seen from Text-fig. 3, the third and fourth extracts are more active than the first and second. This might be taken to indi-

# Serial extraction of desiccated C.T. I













Composite chart of 7 experiments

Extract inoculations				Residue inoculations			
Extracts	No of inoculations	Percent tumors	Average size of	Residues	No of inoculations	Percent tumors	Average size of
1 <sup>st</sup>	17	47	8 tumors • 07×05 cm	1 <sup>st</sup>	15	93.3	14 tumors • 14×12 cm
2 <sup>nd</sup>	17	100	17 • 16×12 •	2 <sup>nd</sup>	14	92.8	13 • 18×15 •
3 <sup>rd</sup>	16	93.7	15 • 19×14 •	3 <sup>rd</sup>	14	100	14 • 20×16 •
4 <sup>th</sup>	16	93.7	15 • 18×14 •	4 <sup>th</sup>	14	100	14 • 28×19 •
5 <sup>th</sup>	16	37.5	6 • 11×09 •	5 <sup>th</sup>	13	100	13 • 25×22 •
6 <sup>th</sup>	16	25	4 • 09×07 •	6 <sup>th</sup>	14	100	14 • 26×21 •
7 <sup>th</sup>	10	—	—	7 <sup>th</sup>	10	100	10 • 23×17 •
8 <sup>th</sup>	10	—	—	8 <sup>th</sup>	10	100	10 • 24×20 •

TEXT-FIG. 1. In this series of experiments and those included in subsequent text-figures, the inoculations were made intradermally, each animal receiving from 6 to 8 inoculations. The measurements of the tumors of each fowl used in the charts were those made when the tumor from the control inoculations, or a selected one of the test tumors, had reached a certain size. This method gives more accurate data on the relative potency of the materials tested and largely eliminates the confusing variation due to differences in susceptibility in individual chickens

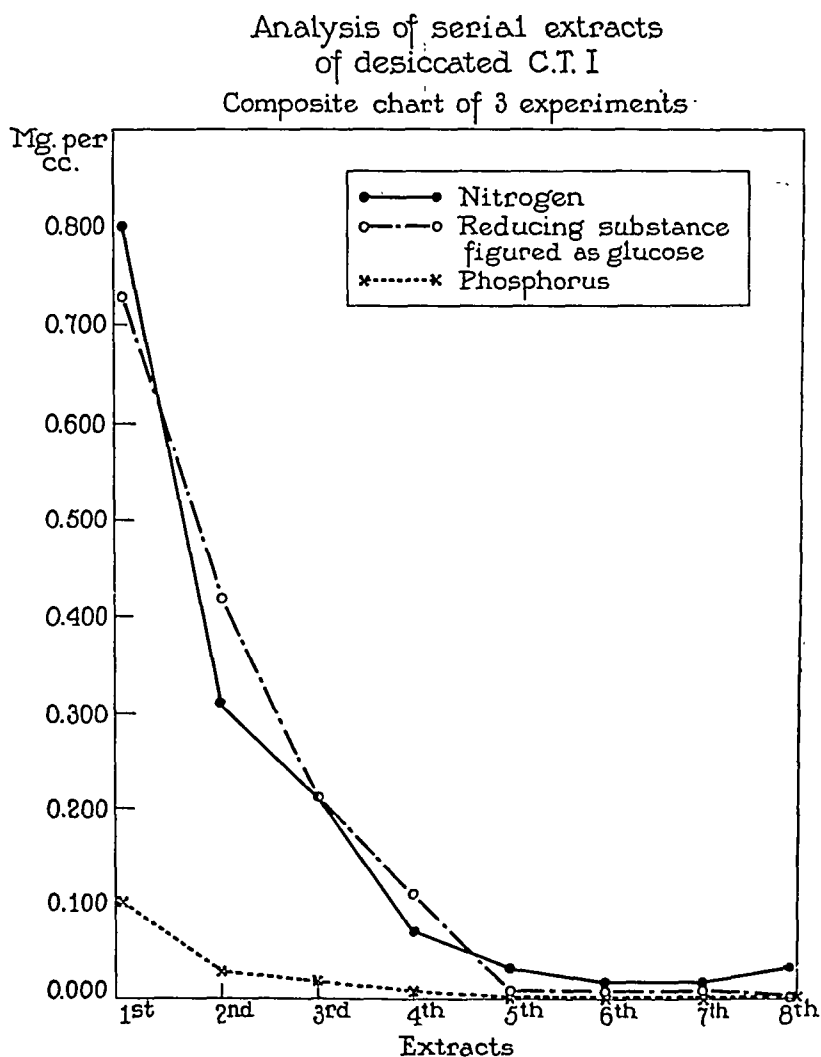
## Serial extraction of desiccated C.T. I

Experiment 6

Extract inoculations			Residue inoculations		
1 <sup>st</sup>	—	No tumor	1 <sup>st</sup>		1.6 × 1.4 cm.
2 <sup>nd</sup>		1.6 × 1.2 cm.	2 <sup>nd</sup>		1.8 × 1.5 •
3 <sup>rd</sup>		2.1 × 1.8 •	3 <sup>rd</sup>		2.0 × 1.6 •
4 <sup>th</sup>		2.0 × 1.5 •	4 <sup>th</sup>		2.3 × 1.6 •
5 <sup>th</sup>		0.8 × 0.6 •	5 <sup>th</sup>		2.6 × 2.3 •
6 <sup>th</sup>	—	No tumor	6 <sup>th</sup>		3.0 × 2.1 •
7 <sup>th</sup>	—	• •	7 <sup>th</sup>		2.6 × 1.6 •
8 <sup>th</sup>	—	• •	8 <sup>th</sup>		2.3 × 1.9 •

TEXT-FIG. 2. For method of inoculation and comparative measurements see explanation of Text-fig. 1.

cate that the active principle is difficultly soluble and that more comes out with the repeated washings; but the fact that the residues after extraction become progressively more active leaves little doubt



TEXT-FIG. 3

that some inhibiting substance is being removed with the extract. That the first extracts contain considerable amounts of the active principle is shown by the next experiments.

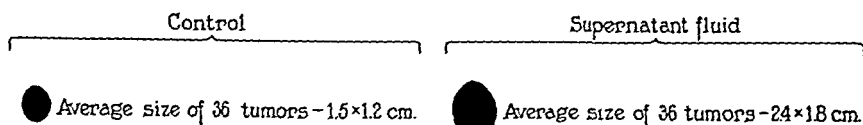
*Removal of Inhibitor by Adsorption on Aluminum Hydroxide*

In a preceding paper the results of treating extracts of the chicken tumor with aluminum hydroxide have been described in connection with purification of the tumor agent (5). The removal of approximately 90 per cent of the nitrogen-containing elements from the extract with the aluminum hydroxide was accomplished without loss of activity in the remaining fluid. In fact, the remaining fluid was more active than the original extract, in spite of the removal of a certain amount of the agent on the aluminum hydroxide adsorbate.

*Method.*—A concentrated Berkefeld filtrate of fresh chicken tumor or an extract of tumor desiccate was added to an equal volume of aluminum hydroxide (Willstätter Type C), prepared in the usual way (6). This was shaken until thoroughly mixed,

Effect of treatment with aluminum hydroxide  
on the activity of C.T. I extract

14 experiments













TEXT-FIG. 4. The method of recording measurements of tumors was the same as that used in the preceding text-figures. The control inoculation was a sample of the same tumor extracts subsequently treated with aluminum hydroxide.

centrifuged and the supernatant fluid drawn off. Chickens were inoculated intradermally with 0.2 cc. of this fluid in several areas, and also with equal amounts of the original extracts for controls. Weekly measurements were made. The average of the results of 14 experiments, in which 36 inoculations were made of both tumor extract and aluminum supernatant fluid, are shown in Text-fig. 4.

The fact that an appreciable amount of the agent is removed with the aluminum has been shown in a previous paper. In spite of this loss in concentration the fluids left after adsorption on aluminum are markedly more active in the production of tumors than the full extracts before adsorption. This seems to indicate that an inhibiting substance must have been carried with the aluminum fraction, leaving the reduced concentration of the agent in the supernatant fluid more active, unhampered by an inhibitor.

*Inhibiting Substance in Slower-Growing Tumors*

While the Chicken Tumor I is extremely rapid in its growth, occasionally a slower-growing tumor is encountered, or the tumor appears

Material inoculated	Inhibitors from chicken tumor I				Average size of tumors
	No. of inoculations	Per cent inhibited	No. of tumors		
Heated extract of slow-growing tumor + active filtrate	50	78	11		0.9 × 0.7 cm.
Active filtrate (alone)	21	0	21		20 × 13 "
Heated extract of slow-growing tumor + aluminum supernatant fluid	33	91	3		0.8 × 0.8 "
Aluminum supernatant fluid (alone)	24	0	24		25 × 18 "
Heated extract of rapid-growing tumor + active filtrate	37	0	37		1.9 × 1.7 "
Active filtrate (alone)	26	0	26		2.2 × 1.8 "
Heated extract of rapid-growing tumor + active aluminum supernatant fluid	9	0	9		1.8 × 1.4 "
Aluminum supernatant fluid (alone)	6	0	6		1.6 × 1.3 "
Heated mucoid exudate from slow-growing tumor + active filtrate	14	100	0	—	
Heated mucoid exudate from rapid — growing tumor + active filtrate	10	0	10		2.0 × 1.3 "
Active filtrate (alone)	20	0	20		1.9 × 1.3 "

TEXT-FIG. 5. The figures given here are based on 250 inoculations. Those included in the inhibitor group were inoculations resulting in no growth. There is undoubted evidence of retardation even when tumors did arise from these test inoculations. The system of recording measurements of tumors was the same as that used in the preceding charts.

at times to pass through a phase of reduced malignancy. It was considered possible that these phases might be due to relative variations in the ratio of agent and inhibitor.

*Experiments.*—The desiccates of a number of slower-growing tumors were used in these experiments. An extract was prepared in the usual way, and the tumor-producing activity was destroyed by heating at 55°C. for 30 minutes. This inactivated material was mixed with an equal amount of an active extract from a fast-growing tumor, and 0.4 cc. injected intradermally into chickens. As a further test the action of the inactivated extract was tested on a highly potent fluid left after adsorption on aluminum hydroxide. The results of some 35 experiments, in which 250 test inoculations were made, are shown in Text-fig. 5.

The heated extract of slow-growing tumors completely neutralized the tumor-producing power of the active extracts in 78 per cent of cases, and partly neutralized the activity in the remaining 22 per cent. The more active supernatant fluid from tumor extracts treated with aluminum, inoculated together with the inhibiting extract, failed to induce tumors in 91 per cent of tests. The control injections of active extracts and aluminum supernatant fluid resulted in 100 per cent tumors. The mucoid exudate obtained from certain slow-growing tumors, showed after heating a similar inhibiting action on active extracts. No inhibiting action was noted with the heated extracts or the mucoid exudate obtained from rapidly growing tumors.

All attempts to release the inhibiting substance in detectable amounts after adsorption on aluminum hydroxide have thus far failed. Berkefeld filtrates of fresh tumors show little evidence of the presence of an inhibitor, owing perhaps to the relatively great dilution of this material.

The effect of heat on the inhibitor was next tested. The same methods were used as in the foregoing experiment, except that various samples of the extract were heated at 60°, 65°, 70°, 75°, 80°, 90° and 100°C. for 30 minutes each. The effect of these samples was tested on the tumor-producing power of an active extract. The results, as shown in Table I, demonstrated that little or no inhibiting power remains in the specimens heated to 65° and over.

From the above experiments it is evident that the slow-growing chicken tumors contain an inhibiting factor capable of neutralizing the tumor agent in its most active form. The inhibiting substance withstands 55°C. for 30 minutes, but is inactivated when heated above 65°C. The fact that the more active tumors do not contain sufficient amounts of the inhibitor to be demonstrable by this method, although



there is adequate proof that it is present, indicates that the degree of malignancy may depend in part on the ratio of agent to inhibitor. There is no doubt that the individual susceptibility of the inoculated fowl plays a part; but, when a number of extracts of different tumors of the same type are injected in the same fowl, the variation in potency is evident. If, as we have noted many times, this test is repeated on a number of chickens, some will be markedly more susceptible than others; but the relative activity of the different extracts will be manifest in all.

TABLE I  
*Effect of Heat on the Chicken Tumor Inhibitor*

Material inoculated		No. of inoculations	No. positive	Positive <i>per cent</i>
Active tumor extract plus.....	Inhibitor heated 30 min. at 55°C.....	8	2	25
	“ “ 30 “ “ 60° “ .....	8	2	25
	“ “ 30 “ “ 65° “ .....	8	3	37.5
	“ “ 30 “ “ 70° “ .....	8	7	87.5
Active extract alone (control).....		8	8	100
Aluminum supernatant fluid of active extract plus	Inhibitor heated 30 min. at 55° C.....	3	0	0
	“ “ 30 “ “ 60° “ .....	3	2	66.6
	“ “ 30 “ “ 65° “ .....	3	3	100
	“ “ 30 “ “ 70° “ .....	3	3	100
	“ “ 30 “ “ 75° “ .....	3	3	100
	“ “ 30 “ “ 80° “ .....	3	3	100
	“ “ 30 “ “ 90° “ .....	3	3	100
	“ “ 30 “ “ 100° “ .....	3	3	100
Aluminum supernatant fluid of active extract alone (control)...		6	6	100

#### DISCUSSION

From the point of view of the suggested hypothesis, according to which the tumor agent may be related to the normal growth-controlling mechanism of the cell, it might be expected that an inhibiting agent would also be present in the tumor. This is suggested by the fact that biological forces are generally balanced phenomena, the presence of an active force checked by a retarding one. There seems little doubt from the results reported here that an inhibitor does exist in the chicken tumors studied, more powerful in the extracts of slow-growing tumors, but definitely present in those of more rapid development.

Aside from individual variation in susceptibility of the fowls, the relative activity of any given tumor extract seems to depend on the proportion of agent to inhibitor. It is not unusual to have an inactive extract, which, after removal of something by adsorption on aluminum hydroxide, shows the presence of sufficient agent to produce vigorous tumors. These observations, taken with the fact that the inhibitor from the chicken tumor acts definitely on mouse sarcoma and is without effect on carcinoma (7), suggest that this agent is a specific factor, not an incidental proteolytic enzyme or accidentally injurious chemical substance.

The relationship of this inhibitor to the normal growth-balancing mechanism of cells is not established by the experiments reported here. Theoretically, if our hypothesis is correct, it should be possible to separate the inhibitor from active normal tissues, just as it should be possible to isolate the growth-stimulating agent. While there is evidence that the latter can be accomplished,<sup>2</sup> the methods thus far used have not yielded regular results. That an inhibiting substance can be secured from normal tissues for mouse tumors under certain conditions is established.<sup>3</sup> Perhaps these results with the chicken tumor agent, deemed to represent an adsorption on normal tissues *in vitro*, really represent neutralization by an inhibitor (8). The relationship of the inhibitor in the tumor to the "antibody" which Andrews has demonstrated in the blood of chickens with slow-growing tumors has not yet been determined (9).

While the presence of an inhibiting substance in the chicken tumor is established, and it would appear to be a specific force, its true nature and its relationship to the causative agent on the one hand, and to the

<sup>2</sup> In a report to the International Cancer Conference, London, 1928, a reference was made to tumors induced by the injection of a fraction of an extract of normal chicken testicle. 4 experiments thus far have resulted positively and twenty-three tumors have been produced by this method. However, there have been many negative experiments. Whether these results indicate that the method is inadequate, giving only occasionally the growth factor in sufficient concentration or free enough from the hypothetical inhibitor to induce tumors, or whether there is some other explanation, are questions which cannot be answered at present.

<sup>3</sup> A preliminary report has been published in *Science*. The complete study will appear later in *The Journal of Experimental Medicine*.

balancing factor of normal cells on the other, are questions which must await further development.

#### SUMMARY

The presence of an inhibiting substance in the chicken tumor is shown by the fact that a desiccate of the tumor is more active after it has been washed two or three times with water, and that an extract of the tumor is more potent after some factor is removed by adsorption on aluminum hydroxide.

When the tumor-producing factor in an extract of a slow-growing tumor has been destroyed by heating at 55°C. it is found to have the property of neutralizing a highly active tumor extract. This inhibiting property is destroyed by heating over 65°C.

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# PROPERTIES OF THE CAUSATIVE AGENT OF A CHICKEN TUMOR

## V. ANTIGENIC PROPERTIES OF THE CHICKEN TUMOR I\*

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In the early investigations into the nature of the etiologic agent of fowl tumors, immunity phenomena were given some attention. The first observation was that the manifestations of resistance to these tumors in fowls, whether natural or acquired, are similar to those established for mammalian tumors, the principal feature being a local cellular reaction about the introduced graft (1). As was also established for mammals, the transfer of large amounts of blood or serum from resistant animals had no influence on the growth of established tumors in susceptible ones. Later results indicated that two types of immunity exist, one directed against the etiologic agent as distinct from that directed against the malignant cell (2). That directed against the agent of each kind of tumor seemed to be more specific (3). Rous, Robertson and Oliver (4) undertook to develop antibodies in other species by the injection principally of finely ground tumor tissue or of the blood of fowls in the last stages of the disease. Rabbits failed to show any neutralizing antibodies, but the sera of the injected geese did inactivate the agent and prevent the induction of tumors in chickens. Later Mottram (5) found evidence of antibodies in a few fowls in which tumors had retrogressed. Mueller (6), using cell suspensions of the chicken tumor, was able to induce antibodies in rabbits and ducks, while Gye and Purdy (7) working with tumor filtrates have reported on protective antibodies induced in ducks and goats. Andrewes (8) has found natural inhibitors for the tumor agents in the blood of chickens with some evidence that such "antibodies" develop in the blood of chickens with a slow-growing fibroma which are effective against the agents of more malignant tumors.

The studies to be reported here were undertaken along with attempts to isolate and purify the active principle of the chicken tumor (9),

\* This investigation was carried out under the Rutherford Donation.  
† Fellow of the Rockefeller Foundation.  
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their primary object being to obtain light on the nature of the tumor agent through a better understanding of its antigenic properties.

*Method.*—When the source of the agent was fresh tissue, 25 gm. of tumor were ground with sand and extracted with 500 cc. of fluid. After passage through a Berkefeld filter, the extract was concentrated to 1/5 of its original volume in alundum thimbles lined with 8 per cent soluble cotton membrane. When a desiccate of the tumor was the source, 1 gm. of the powder was extracted with 60 cc. of the fluid. After centrifugation the extract was filtered through paper.

Rabbits were used throughout. The general method of immunization was to give 5 cc. of the antigen intravenously at 2 day intervals until each animal had received 6 injections or 30 cc. of the antigen. From 12 to 14 days after the last injection the animals were bled from the heart and the serum collected. The precipitating and neutralizing power of the sera were tested within a few hours after withdrawal of the blood. The precipitin tests were run with dilutions of the antigen to 1:1 to 1:320. For the neutralizing power of the sera, chickens were injected intradermally with a mixture of 0.5 cc. of serum to 0.2 cc. of a concentrated fresh tumor extract with control injections of tumor extract alone and with normal rabbit serum.<sup>1</sup>

### *Comparison of Antigenic Properties of Tumor Extracts and Protein Fractions of Extracts*

In the first group of tests the antigenic properties of chicken tumor filtrates, prepared by three different methods of extraction, were compared with the acid-precipitable protein (10) of each extract.

The usual method of preparing tumor filtrates is to extract the tumor with Ringer's solution or normal saline solution. In our experience, however, the results have been somewhat better if the tissue is extracted with distilled water, keeping the suspension slightly alkaline during the procedure. A third method used, with the expectation of securing more of the nuclear protein in the solution, was to extract the tumor material with 5 per cent salt solution.

A given amount of each extract was divided into two equal portions, one to be used for testing the antigenic properties of the extract as such, and the other as the source of the protein fraction. The latter was secured by the addition of N/10 lactic acid until a clear-cut precipitate was formed. The point at which this occurred varied somewhat with different extracts, but was generally between pH 4 and 4.4. The precipitates were washed with distilled water and then dissolved

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<sup>1</sup> The neutralizing power of the sera was tested in all experiments within a few hours of the withdrawal of the blood from the immunized rabbits. Therefore our tests do not indicate whether or not complement is necessary for the reaction.

in N/200 NaOH and sufficient water added to bring the volume up to that of the original extract. The supernatant fluids, after removal of the precipitate, were adjusted to pH 7.2 and also used as antigens.

The material tested in this experiment included the following: water, Ringer's solution and 5 per cent salt solution extracts of chicken tumors, their acid-precipitable protein fractions and the supernatant fluid after removal of the precipitate. The tumor-producing property of such extracts and precipitates, as tested on chickens in each experiment, is shown in Table I. In 12 experiments the antigenic properties were determined by the injection of 48 rabbits. In each case the precipitating and neutralizing powers of the antisera developed were tested. The results are shown in Text-figs. 1 and 2.

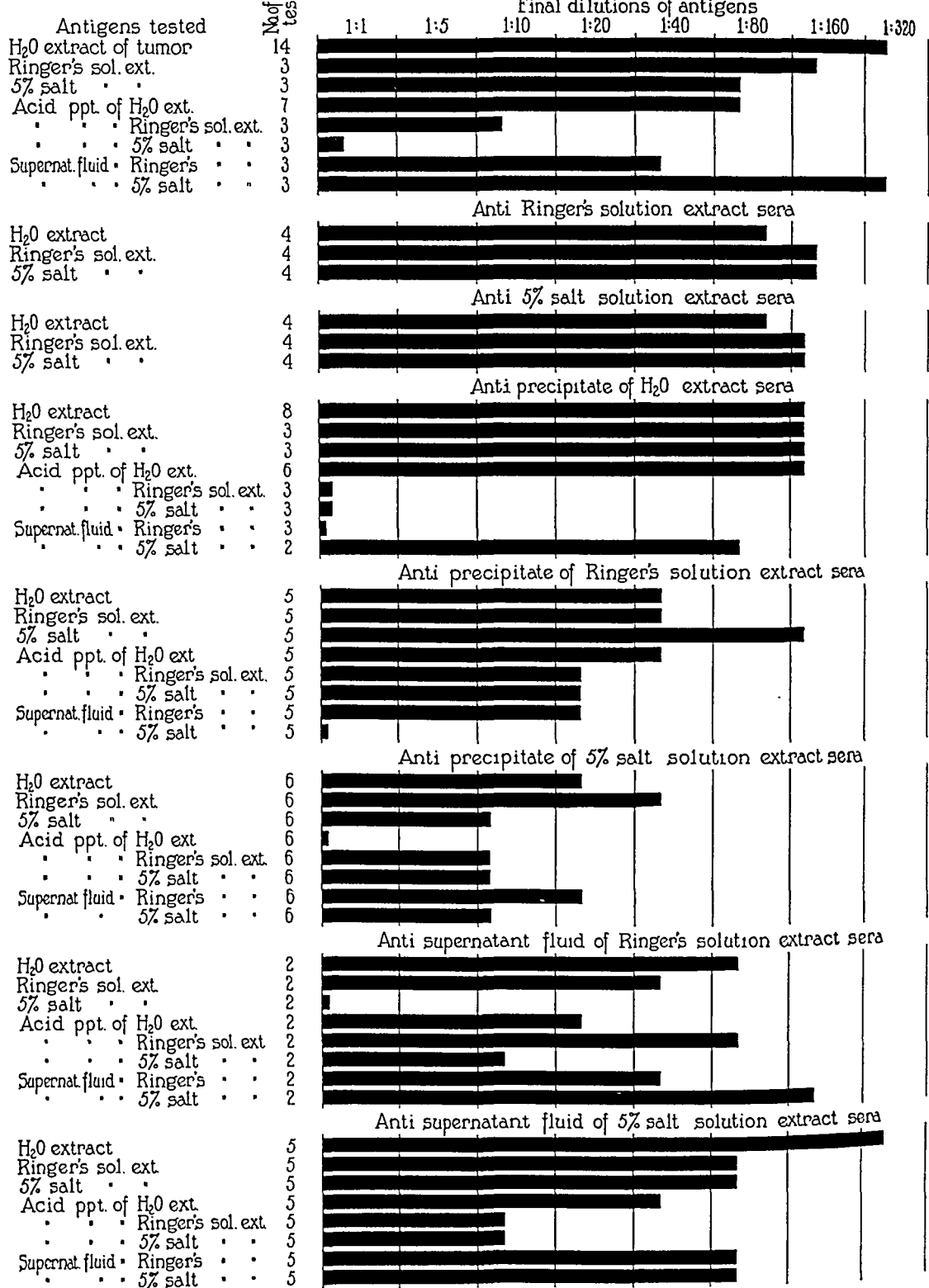
TABLE I

Material inoculated	From fresh tumor		From desiccated tumor	
	No. of tests	Tumors	No. of tests	Tumors
		<i>per cent</i>		<i>per cent</i>
Water extract.....	8	100		
Protein fraction of water extract.....	14	100	14	100
Supernatant fluid after precipitation.....	12	0	11	91
Ringer's solution extract.....	12	92	8	0
Protein fraction of Ringer's solution extract..	14	21	8	100
Supernatant fluid after precipitation.....	12	0	11	27
5 per cent salt extract.....	12	0	8	0
Protein fraction of salt extract.....	14	25	8	0
Supernatant fluid after precipitation.....	12	0	11	100*
		0	8	0

\* The tumors in this group were only a fraction of the size of those developing from the injection of the other extracts.

As a control to these tests, rabbits were immunized in the same fashion with water extracts of chicken muscle, liver, kidney, testicle and nucleoprotein of the blood, 2 rabbits being used for each preparation. The antisera to liver, kidney and testicle showed slight precipitins for tumor extracts, but there was no evidence of these bodies in the other sera. With the possible exception of the anti muscle and anti testicle sera, there was no evidence of neutralization of the tumor agent in the protection tests. The figures for these two may be significant, but compared with the definite evidence obtained with the antisera developed against the tumor extracts the results with them are not striking.

The significant points shown by this group of experiments are that precipitating antibodies may be developed in rabbits against a water or Ringer's solution extract of chicken tumors, and that the protein frac-



TEXT-FIG. 1. The amount of extension of the lines into the spaces under the dilutions indicates the intensity of the precipitin reaction for the given dilution of antigen.

tion carrying the activity of either of these extracts is equally effective in calling out precipitins in rabbits. The neutralizing power is shown not only by the figures for complete inhibition but also by those for partial inhibition, as indicated by the size of the tumor in the positive inoculations. The results with 5 per cent salt solution are significant

Neutralization of tumor extracts by anti sera

Sera inoculated with tumor filtrate	No. of sera tested	No. of inoculations	Percent neutralization	No. of tumors	Average size of tumors
Anti H <sub>2</sub> O tumor extract	10	34	82.4	6	● 0.9 × 0.6 cm.
Anti Ringer's solution, extract	4	10	90.0	1	● 1.3 × 1.2 "
Anti 5% salt solution extract	4	10	40.0	6	● 1.0 × 0.8 "
Anti precipitate of H <sub>2</sub> O extract	7	30	76.7	7	● 0.8 × 0.8 "
Anti precipitate of Ringer's solution extract	7	20	80.0	4	● 1.0 × 0.8 "
Anti precipitate of 5% salt solution extract	6	20	25.0	15	● 1.9 × 1.5 "
Anti supernatant fluid of Ringer's solution extract	4	9	33.3	6	● 1.5 × 1.0 "
Anti supernatant fluid of 5% salt solution extract	5	11	45.5	6	● 1.9 × 1.5 "
Normal rabbit serum	12	39	2.6	38	● 2.3 × 1.4 "

TEXT-FIG. 2. All inoculations were made intradermally and each fowl received besides the test inoculations an injection of untreated tumor extract for control. The measurements were recorded when the tumor from the control inoculation had attained a certain size.

only because there is some evidence that neutralizing antibodies may be developed by extracts which themselves have a low grade tumor-producing activity. The same lack of relationship between the tumor-producing properties of the antigen and antibody response is seen in the fact that the neutralizing power of the sera developed against the protein precipitate of a Ringer's solution extract is just as effective



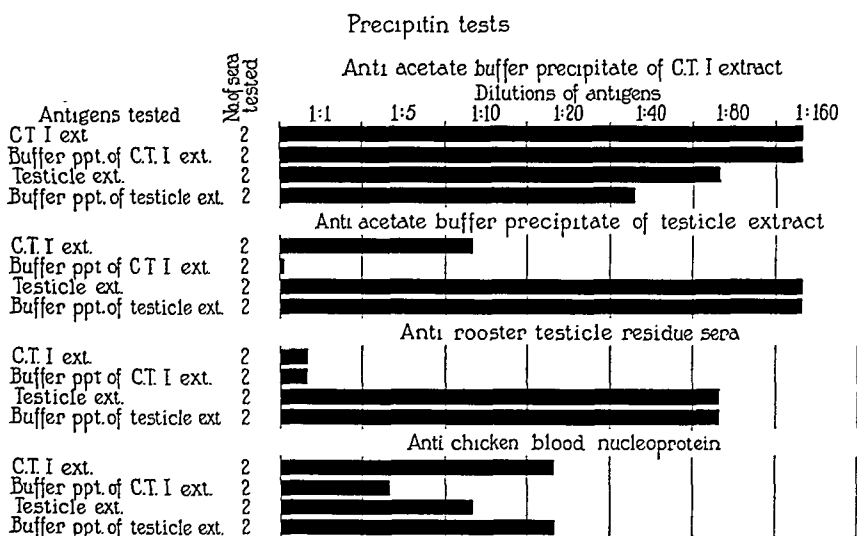
as that of the sera developed against the precipitate of the water extracts. Yet these two antigens show a marked difference in tumor-producing power. The antisera developed against normal chicken tissue extracts have no very significant precipitating or neutralizing antibodies toward the tumor extract.

### *Antigenic Properties of Buffer Precipitates of Tumor Extracts*

As another step in the attempts to isolate the agent from the tumor extracts, it was hoped that a clearer-cut separation could be obtained if the precipitation were carried out with buffered solutions (10). The study in parallel of the antigenic properties of these precipitates is recorded here, primarily because of the results with normal tissue extracts which are used as controls.

*Experiment.*—The water extracts of dried tumor and of fowl testicle were prepared as described above. To secure the precipitates, these extracts were added in the ratio of 1 cc. to 5 cc. of M/100 acetate buffer at a pH of about 4.2. The precipitates were dissolved in N/200 NaOH. The residues, after extraction of the testicle material, were suspended in Ringer's solution and also used as antigens. As a further control the nuclear material from chicken red blood cells was obtained by laking the blood and washing out the hemoglobin. Thus the antigens tested in this experiment included buffer precipitates from the water extracts of tumor and testicle tissue, the water-soluble testicle residue and the nucleoprotein of the chicken blood. 2 rabbits were used for each antigen. The results of precipitin and neutralization tests are given in Text-figs. 3 and 4.

It is of interest to note that the sera developed against the tumor extract precipitates had precipitins for both testicle extract and its precipitate, while the anti testicle precipitate and testicle residue sera showed no reaction with the tumor precipitate. The antibody response to the blood protein was slight, but about equal for the tumor and testicle extracts. The neutralizing power of the anti tumor sera is definitely shown by the number of negative results from inoculation with the sera and fresh tumor extracts, as well as by the small size of the tumors in successful inoculations. The other sera were without definite neutralizing power, with the possible exception of that developed against the testicle residue, which yielded when injected with an active extract 33 per cent of negative inoculations, while the tumors



TEXT-FIG. 3. The lines show the extent of the principal reaction. The degree of penetration into the spaces for the dilutions indicates the intensity of the reaction for that particular dilution.

#### Neutralization of tumor extracts by anti sera

Sera inoculated with tumor extract	No. of sera tested	No. of inoculations	Percent neutralization	No. of tumors	Average size of tumors
Anti buffer precipitate of tumor extract	2	6	666	2	● 11 × 08 cm.
Anti buffer precipitate of testicle extract	2	14	71	13	● 17 × 14 .
Anti testicle residue	2	6	333	4	● 10 × 08 .
Anti chicken blood protein	2	14	71	13	● 17 × 14 .
Normal rabbit serum	3	9	00	9	● 24 × 19 .
Tumor extract (control)	—	10	—	10	● 18 × 15 .

TEXT-FIG. 4. The method of recording the relative sizes of the tumors in these experiments was the same as that described under Text-fig. 2.

from the positive inoculations were somewhat smaller than the controls.

*Antigenic Properties of the Purified Tumor Agent*

The various attempts to determine the nature of the tumor agent resulted in a method of securing very active products with low protein content or even almost free from protein. The first observation in this line was that a water extract of dry tumor had a relatively low tumor-producing activity. The residue extracted a second time gave a more active material, while a third extract of the material was still more potent in the production of tumors (11). The principal significance of this observation was that over 60 per cent of the soluble nitrogen-containing compounds were removed in the first extract, while the most active extract, the third, had less than 12 per cent and the fourth, which was still very active, contained only about 0.08 mg. of nitrogen per cc.

Another method of removing the bulk of incidental protein in the tumor extracts is by adsorbing them out on colloidal aluminum hydroxide (Willstätter Type C). With the proper ratio between the amount of aluminum hydroxide and tumor extract it has been found that practically all of the proteins are taken down with the colloid, leaving a highly active material in the supernatant fluid. The details of this method are given in another paper (12). The fact that guinea pigs are not sensitized by this material indicates the extremely small amount of protein remaining. The principal contamination of this supernatant fluid proved to be a material resembling chondroitin-sulfuric acid. This can be removed by combining it with a basic protein and then precipitating out the new compound without reduction in the tumor-producing activity. The principal products developed in this study were investigated for their antigenic properties.

*Experiment.*—In this group of experiments the antigenic properties of the following products were investigated: first and third extracts of tumor desiccate, the supernatant fluid after adsorption of a tumor extract with aluminum hydroxide<sup>2</sup> (which will be referred to as aluminum supernatant), the supernatant fluid of the above extract after precipitating out the chondroitin material with gelatin (which will be called the gelatin supernatant), and finally the chondroitin-gelatin precipitate. The system of injecting the rabbits was the same as that used in the

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<sup>2</sup> The aluminum supernatants used in these experiments were prepared by Dr. O. M. Helmer

## Neutralization of tumor extracts by anti sera

Material inoculated	No. of sera tested	No. of inoculations	Per cent negative	No. of tumors	Average size of tumors
Anti tumor extract serum + tumor extract	10	32	53.2	15	● 12 × 10 cm.
Anti tumor extract serum + aluminum supernatant fluid	8	20	700	6	● 0.9 × 0.8 .
Anti aluminum supernatant fluid serum + tumor extract	14	68	73.6	18	● 0.8 × 0.6 .
Anti aluminum supernatant fluid serum + aluminum supernatant fluid	14	65	84.6	10	● 0.8 × 0.7 .
Anti 3 <sup>rd</sup> extract serum + tumor extract	4	15	66.6	5	● 0.9 × 0.8 .
Anti 3 <sup>rd</sup> extract serum + aluminum supernatant fluid	4	9	77.7	2	• 0.4 × 0.3 .
Anti gelatin supernatant fluid serum + tumor extract	15	29	24.1	22	● 1.9 × 1.3 .
Anti gelatin supernatant fluid serum + aluminum supernatant fluid	6	15	6.6	14	● 1.9 × 1.4 .
Anti gelatin precipitate serum + tumor extract	12	34	11.7	30	● 1.8 × 1.4 .
Anti gelatin precipitate serum + aluminum supernatant fluid	6	17	0.0	17	● 2.0 × 1.2 .
Normal serum + tumor extract	13	29	0.0	29	● 2.3 × 1.8 .
Normal serum + aluminum supernatant fluid	10	21	0.0	21	● 2.3 × 1.7 .

TEXT-FIG. 5. For explanation of tumor measurements see Text-fig. 2

preceding experiments. The activity of each product used in the immunization was tested on chickens.

Tests showed that the sera from animals immunized with the full tumor extract had precipitins for the extract as such and gave a doubtful reaction with the third

extract, but no evidence was obtained of precipitins for the aluminum supernatant and gelatin supernatant fluids. The antisera for the third extract gave a doubtful reaction with the third extract and had no precipitins for the extract as such or any of the other preparations. The sera of animals injected with aluminum supernatant, gelatin supernatant and gelatin precipitate showed no precipitins for any of the preparations, nor did these sera give flocculin when tested with the Ramon technique. The results of the neutralization test are shown in Text-fig. 5.

In 10 experiments the complement-fixing power has been tested on 11 sera developed against the water extract of the chicken tumor, 21 sera developed against the supernatant fluid of a tumor extract after the major portion of the protein had been adsorbed out on aluminum hydroxide, 9 sera from rabbits injected with extracts after the removal of the viscous material and 9 against the viscous precipitate. The method employed in the immunization was that described above, and the standard Wassermann method was used for determining the complement fixation.

The experiments suggest that the precipitins in the sera developed against the tumor extract have no direct relation to the neutralizing property. The basis for this statement is to be found in the fact that these sera fail to produce precipitation or flocculation in the highly active aluminum supernatant fluid of a tumor extract. Furthermore the antisera developed against this active material showed no precipitins with the aluminum supernatant fluid or the full tumor extract, although they have a high neutralizing power for the tumor agent. The sera of the rabbits injected by Rous, Robertson and Oliver with the tissues of the chicken tumor had strong precipitins for chicken serum, yet had no evident effect on the tumor-producing agent (13). Altogether it would seem that the precipitins result from the injection of incidental proteins of the tumor not directly associated with the tumor-producing agent. The failure in practically all of the tests of the most highly purified product to induce either precipitins or antibodies for the tumor agent may require further investigation. As this material has a tumor-producing activity at least equal to that of the full tumor extract, it does not seem probable that the absence of antibody response can be attributed to the failure to inject into the rabbit sufficient tumor agent.

#### *Complement Fixation Tests with Anti Chicken Tumor Sera*

In addition to the tests of the rabbit sera for precipitins and for neutralizing power, the presence of complement-fixing antibodies has been investigated.

Each serum was tested in two amounts, 0.2 cc. and 0.1 cc., against undiluted water extracts of chicken tumor and aluminum supernatant fluid, and also with these two antigens diluted 1:10 and 1:5.

The 11 anti tumor extract sera, in both amounts tested against the undiluted extract as antigen, gave complete fixation, but there was no fixation with this antigen when diluted or with the undiluted or diluted aluminum supernatant fluid used as antigen. The 21 anti aluminum supernatant fluid sera gave no fixation with aluminum supernatant fluid, but did with undiluted water extract of the tumor. The anti gelatin supernatant fluid sera and anti gelatin precipitate sera gave no fixation with the aluminum supernatant fluid, but 4 out of 9 of the former and 3 of the latter did give fixation with the undiluted water extract of the tumor.

It is evident from these results that there is not a sufficient amount of the antigenic factor present in the aluminum supernatant fluid to interact with the antibody and fix the complement. Yet, by the only test available, namely tumor production in chickens, the concentration of the tumor agent in the aluminum supernatant fluid is almost equal to that in the tumor extract as such. Therefore, it would seem that the antibody against the tumor agent is not demonstrable by the complement fixation test.

#### DISCUSSION

The interpretation of the results reported here offers some difficulties, in that there is no very close analogy with the antigenic properties of known disease-producing agents. It seems plain that the precipitins stimulated in rabbits by the injection of the intact tumor extracts are developed against the incidental proteins of the tumor and have no essential association with the antibodies capable of neutralizing the tumor-producing activity of the etiologic agent. The most effective neutralizing sera were those developed against the purified agent practically free from protein, and these sera showed no demonstrable precipitins or complement-fixing antibodies. This suggests the type of immune bodies developed against certain toxins and recalls the discussion as to whether precipitins in antitoxic (14) and anti enzyme sera are not incited by contaminating proteins. In the latter case the question seems to be answered by the work of Kirk and Sumner (15) who have found a parallel development of precipitins and neutralizing antibodies to crystalline urease, a result which might have been expected as the enzyme in this case is a protein according to these authors.

The failure of the more highly purified agent to induce any demonstrable antibodies, in our opinion, is probably not attributable to the reduction in concentration. There is no doubt that some of the agent is lost in each step in purification, but this loss must be small for there is no evidence of reduction in tumor-producing power, a property which is definitely influenced by comparatively slight dilution. An analogy suggested by this result is the failure of the purified specific substance of pneumococci to induce antibodies, but this substance is definitely precipitable in even very high dilutions with the antisera developed to the type pneumococci (16), while with the purified tumor agent there is no such reaction with the sera of animals immunized with the full tumor extract.

In contrasting the antigenic properties of the chicken tumor agent with those of the viruses, perhaps the most striking difference is the comparative ease with which neutralizing antibodies for the tumor agent can be developed in non-susceptible species and the doubtful results obtained with most of the viruses under similar conditions. In fact with many viruses, notably vaccine virus, protective antibody development is not only limited to susceptible species but it is doubtful if they develop then in the absence of an actual infection with manifest lesions of the disease (17).

#### SUMMARY

The injection of tumor extracts and their active protein fractions into rabbits induced the formation of precipitins and neutralizing antibodies. When the major portion of proteins in the tumor extract had been eliminated it induced the formation of neutralizing antibodies, but not of precipitins. The tumor agent, more highly purified by removal of the viscous fraction, did not induce precipitins, and only 2 out of the 15 sera gave any evidence of neutralizing bodies. After the removal of the major portion of protein, the extracts showed insufficient interaction with the sera to fix complement.

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## TYPHUS FEVER

### IV. FURTHER OBSERVATIONS ON THE BEHAVIOR OF RICKETTSIA PROWAZEKI IN TISSUE CULTURES

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#### PLATE 6

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In a previous article (Paper III of this series) (1) we described a technic for the propagation and morphological study of *Rickettsia prowazeki* in tissue cultures. Although definite evidence of the multiplication of the organism was presented in this publication, we found that our cultures regularly became non-infectious and histologically *Rickettsia*-free on or before the 12th day *in vitro*, in spite of continued good growth of the cells. In one group of cultures, however, extremely heavy infection of the majority of cells present was found on the 16th and 21st days *in vitro*. Since this group of cultures was incubated at a lower temperature than the others, (30–34°C., instead of 37°C.) a systematic study of the effect of temperature on the multiplication of the organisms was undertaken. These experiments are published in a separate article (Paper V of this series) (2). They show that a temperature of approximately 32°C. is optimum for *Rickettsia* growth under these conditions, and explain our previous difficulty in carrying the infection beyond the 12th day.

#### *Technic*

In addition to the change in the temperature of incubation, the following changes from the technic described in Paper III have been adopted.

1. Extract of normal spleen from a young guinea pig (200 to 300 gm.) has been substituted for embryonic extract.

The spleen is removed aseptically, placed in about 20 cc. of Tyrode's solution and cut into ten or twelve pieces, with the scissors. Most of the blood is washed out by this procedure. The fragments are then transferred to a sterile centrifuge tube and thoroughly crushed by means of a glass rod, having a diameter about the

same as that of the narrowest part of the lower portion of the tube. 10 cc. of Tyrode's solution are then added to the tube and the pulp is thoroughly mixed with the fluid by stirring. After centrifugalization the straw-colored and very slightly cloudy fluid is drawn off and stored at low temperature.

2. The splenic extract described above has been diluted with an equal volume of distilled water before use.

Splenic extract was adopted chiefly because it is easier to make. Cultures grow more slowly than when embryonic extract is used, but remain in good condition without transfer for a longer period of time (up to 21 days when grown at 32°C.). This slowing down of the rate of growth and degeneration undoubtedly favors the multiplication of the organism.

The reduction in tonicity of the extract by adding equal parts of distilled water makes the final tonicity of the cultures about 25 per cent below normal. Evaporation before and after sealing the cultures probably compensates to a large extent for this dilution. After the air in the Petri dishes becomes saturated with moisture at the temperature of incubation, no further evaporation appears to occur.

Staining of sections was done by the Giemsa method.

### *Description of Cultures at Various Ages*

By sectioning a large number of cultures at different intervals after planting, we have obtained data concerning the progress of the infection in cultures growing slowly at 32°C. With the elimination of variables in technic, quite uniform and consistent results have been obtained. In 81 out of 86 cultures sectioned histologically on or after the 10th day *in vitro*, the infection has been so voluminous that distended cells could be easily identified with low power magnification.

*3 to 4 days.*—Cultures of this age always showed less than five infected cells per section. In some instances there may not have been any increase over the number of infected cells present in the original exudate. Since, however, we have frequently found three or four infected cells per section when smears of the exudate showed only a rare infected cell after 15 to 20 minutes' search, it is probable that some multiplication occurs almost from the outset. Most of the infected cells at this stage contained diplobacillary forms like those seen in the original exudate. Cells lightly infected with the thread form of the organism were occasionally found at the growing edge of these early cultures, however. This also indicates that multiplication of the organism has begun, since these thread forms have not been found in the original exudate.

Inoculation of single cultures of this age intraperitoneally into guinea pigs almost invariably produced typical typhus with the characteristic scrotal sac and scrotal involvement. The incubation period (6 to 8 days) was about the same as that of the infections produced by injection of single fragments of exudate immediately after floating in Tyrode's fluid.

*7 to 8 Days.*—In cultures fixed on the 7th and 8th days, infected cells ranged from six to fifty per section and the heavily infected ones often stood out clearly under the low power lens. Under oil immersion, these heavily infected cells were found packed with organisms which stained sharply and fairly deeply, ranging in color from bluish purple to reddish purple. In undifferentiated sections, isolated groups of organisms occasionally were stained blue but usually they were purple, even when the nuclei, cytoplasm and fibrin clot were all a bright clear blue. In these heavily infected cells, the organisms were morphologically like those seen in the original exudate. Many lightly infected cells were present at this stage, however, and in these cells the organisms appeared larger and tended to grow in long chains and in what appeared to be solid threads. Chains of fifteen to twenty individual bacilli were not uncommon. These chains were often curled because they were longer than the cells in which they were enclosed and occasionally measured as much as 30 microns in length. These long chains of relatively large bacillary forms were definitely associated with lightly infected and "healthy" appearing young cells and were most numerous at the growing edge of cultures showing many mitotic figures. Inoculation of these 7 day cultures intraperitoneally into guinea pigs produced a typical reaction. Incubation periods ranged from 3 to 6 days, indicating that *Rickettsiae* were more numerous than in the 3 and 4 day cultures. (The dependence of the incubation period on the number of *Rickettsiae* injected was pointed out in Paper I of this series (3).)

*13 to 18 days.*—The great majority of the cultures of this age group have shown practically unrestricted multiplication of the organism. By this we mean that more than half (often as many as 99 per cent) of the cells in the cultures were infected. Of these infected cells, 10 to 15 per cent were usually so heavily infected that they stood out under low magnification as large, usually rounded, deeply stained, purple structures, ranging up to 60 micra in greatest diameter. They could be clearly seen with a lens magnifying ten diameters. The appearance of these swollen cells, tightly packed with *Rickettsiae* was quite striking (Figs. 1 and 2). In the most distended and heavily infected cells, the individual organisms often could not be resolved centrally but were clearly seen peripherally (Fig. 2). These structures often had the appearance of bacterial colonies but on close inspection it was obvious that their size was limited by the extent to which individual cells could be distended without rupturing. These colony-like structures were often spindle or irregularly star-shaped (corresponding to the shape of growing cells) and even when their position with reference to the cells might otherwise be questioned, their characteristic shape and the fact that they had sharp edges (as though bounded by cell membranes) established their intracellular location.

In cultures of this age, and to a lesser degree in the 7 day cultures, occasional masses of *Rickettsiae* were seen which did not have a sharp edge and were not apparently confined within cell membranes. We interpret these structures as ruptured or autolyzed cells from which the cytoplasm had disappeared or become disseminated, leaving the organisms in a naked mass. It is noteworthy that such free lying masses of organisms almost invariably showed morphological changes which we associate with conditions unfavorable to them. The individual organ-

isms were globular or granular (with marked variation in size but tending to minuteness) and stained more deeply. They also were somewhat more eosinophilic than the vegetative bacillary forms.

Rarely at this stage small clusters of sharply outlined bacilli were found apparently free in the fibrin clot, but such observations were infrequent and organisms were never seen in the fibrin clot at a distance from the tissue. Study of cultures at this stage led to the conclusion that the fibrin clot outside of the cells was a very unfavorable medium for the organism and that its life was brief when it lost the protection afforded by the interior of the cells.

The heavily infected cells at this stage were found chiefly in the central parts of the cultures where growth was slow and these cells usually contained minute forms of the organism with a tendency to granular degeneration in those cells which appeared to be dead or dying. Peripherally the same picture of young healthy cells, lightly infected with larger rods and long chains, was found.

Five guinea pigs inoculated with cultures of this age (13 to 18 days) all reacted typically with incubation periods of 3, 3, 4, 4 and 5 days, indicating that *Rickettsiae* were numerous.<sup>1</sup>

*26 to 36 Days.*—Cultures of these ages (twenty-eight in number) all showed about the same picture as the 13 to 18 day group. In several instances the cells were largely non-viable in appearance (pyknotic nuclei and heavily vacuolated cytoplasm) and the *Rickettsiae* were correspondingly more granular in appearance. In the majority of cultures in this age group, however, mitotic figures were abundant and healthy cells containing rod-shaped *Rickettsiae* predominated. The presence of large numbers of viable *Rickettsiae* at this stage is attested by the fact that three guinea pigs inoculated (from two different groups of cultures) on the 35th and 36th days, reacted in a typical manner after incubations of 3, 2½ and 4 days.

*42 to 52 Days.*—Three cultures have been sectioned on the 42nd, two on the 45th, one on the 51st and four on the 52nd day. In the 42 day cultures, the cells were mostly non-viable in appearance and mitotic figures were very rare. (The growth energy of the cultures appears to decrease after the first 3 weeks, possibly because of the heavy infection, but we think not entirely for this reason.) In these cultures practically every cell was heavily infected but vegetative forms (sharply outlined rods) were in the minority. Many of the cells were without visible nuclei but heavily laden with *Rickettsiae*. One guinea pig inoculated with two 42 day cultures reacted positively and typically with an incubation period of about 48 hours. Smears of the scrotal sac exudate from this animal showed unusually numerous

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<sup>1</sup> An incubation period of 3 days is achieved rarely by inoculation with scrotal sac exudate, only when the entire exudate (about 0.5 cc.) is injected and only when this exudate is unusually rich in organisms. These cultures, having a volume of about 0.3 c.mm. may be assumed to contain about the same number of *Rickettsiae* as 500 c.mm. of the most heavily infected scrotal sac exudate.

*Rickettsia*-filled cells and hundreds of free lying *Rickettsiae* in every field. These *Rickettsiae* appeared unusually large and stained more deeply than usual (an observation which we have repeatedly made in exudates from animals inoculated with cultures after several weeks *in vitro*). The disease was carried for three generations from this animal by inoculation with scrotal sac exudate and no change in virulence or other features was observed.<sup>2</sup>

The two 45 day cultures were in good condition, and mitotic figures were quite numerous. Every cell present was infected and over 50 per cent of the cells were distended with rod-shaped *Rickettsiae*. Inoculation of a guinea pig with two cultures of this age resulted in a strongly positive reaction in about 36 hours (the shortest incubation which we have recorded).

The 51 day culture was in excellent condition at the time of fixation. Sections showed extremely heavy infection of practically every cell. Peripherally, mitotic figures were present in moderate numbers and many cells were packed with long chains of *Rickettsiae*, lying parallel to one another. A guinea pig inoculated with fragments of fibrin clot cut away from the remaining cultures of this age, developed a typical reaction with an incubation period of 4 days. Unfortunately the remaining cultures in this group did not survive the transfer, although they appeared to be in good condition on the 51st day when they were transplanted. 1 week later (on the 58th day) they were found to be non-infectious and histologically *Rickettsia*-free. Apparently the *Rickettsiae* did not survive for as long as 7 days in the absence of living cells.

The four 52 day cultures sectioned all showed extremely heavy infection of practically every cell present. All cells present appeared either dead or dying and no mitoses were observed. Practically all organisms present were in the globular or granular form. Many large masses of free lying organisms were found and there was considerable spreading of the organisms through the fibrin clot. Scattered organisms were present in the fibrin clot even at the distance of a millimeter from the tissue (Fig. 4). These organisms became less numerous, however, as one passed further away from the tissue and we think that they passed out into the fibrin clot simply by diffusion after being set free from the disintegrating cells. In two instances they were heavily concentrated at the line of junction of the old and new fibrin clot but their granular nature in this location made it seem highly improbable that they had multiplied there.

No guinea pigs were inoculated from these 52 day cultures.

#### *Virulence of Organism after in Vitro Cultivation*

By fixing part of the cultures from each group on certain dates and injecting others into guinea pigs, we have been able to compare the

<sup>2</sup> No attempt has been made to conserve the original strain by direct transfer from animal to animal. The strain now in use has been subjected to many *in vitro* periods, ranging up to 51 days in duration. No changes in incubation period or strain virulence have been noted.

morphological picture with the virulence. The accuracy of this method depends upon the degree of variation between individual cultures of the same group, but we have never found marked differences in this respect.

The incubation period is definitely correlated with the number of visible *Rickettsiae* in sections (see Table I in Paper V (2)). In one instance (mentioned above) the injection of two cultures which had been 45 days *in vitro*, resulted in a typical reaction (with marked scrotal swelling) after an incubation period of 36 hours. Two cultures from this same group, fixed on the same day, both showed unusually heavy infection histologically.

In two instances a positive inoculation was obtained from a group which was histologically negative, but the incubation periods were 11 and 14 days. In two instances also, animals which apparently did not react were found immune to subsequent inoculation. Since this same result is occasionally obtained by inoculation with 1 or 2 cc. of blood in routine transfers, it seems reasonable to ascribe it to an insufficient dosage of organisms rather than to altered virulence.

The disease reproduced by injection of infected tissue cultures in the majority of instances differed in no way from that produced by inoculation of fresh scrotal sac exudate. The scrotal involvement was fully as severe and *Rickettsiae* were fully as numerous in the scrotal sac exudate.

In one experiment, however, the disease was reproduced apparently without scrotal sac involvement. A summary of this experiment is given.

Culture Group 81-10 was set out on Aug. 3 in the routine manner and incubated at 32°C. Cultures were washed and reembedded on Sept. 8, Sept. 17 and Sept. 22. Cultures on these various dates were fixed and injected always with positive results. On Oct. 9 (40 days *in vitro*) a single colony remained. The clot was partially liquefied because no transfer had been made for 17 days. The liquid portion of the clot was drawn off in a pipette and amounted to about 0.1 cc. This was divided into five equal parts, four of which were used to inoculate bacteriological culture media (with negative results). The remainder (about 0.02 cc.) was diluted with 4 cc. of Tyrode's solution and injected into a guinea pig. The temperature rose sharply to 105.2° on the 8th day and continued high for 5 days, without scrotal involvement. This animal was then killed. An enlarged spleen was found but the scrotal sac was entirely negative. The gross picture was that characteristic

of Old World typhus. Two guinea pigs inoculated with 4 cc. of heart's blood from this animal both developed typical endemic typhus, with characteristic scrotal reactions and demonstrable *Rickettsiae*.

The lack of scrotal reaction in this instance was probably the result of inoculation with a very small number of *Rickettsiae*, and does not necessarily mean that there was any alteration in virulence.

On the whole the evidence seems conclusive that loss in virulence does not take place up to the 51st day *in vitro*. It seems probable that fully virulent *Rickettsiae* would persist at this temperature as long as the cells could be kept alive. We have had no instance in which infection, once established in a group of cultures (as proven histologically or by animal inoculation) has disappeared from that group except in case of death and disintegration of the cells.

The correspondance between visible *Rickettsiae* and infectivity has been sufficiently complete to rule out the possibility of a virulent invisible form of the organism under the conditions of these experiments.

### *Infection of Normal Tissues in Vitro*

One definite method by which the infection spreads (by mitotic division of infected cells) has been described in Paper III (1). This observation has been repeatedly confirmed and *Rickettsiae* have been found in cells in all stages of mitosis. This method does not suffice, however, to explain infection of practically every cell present (a result which has been obtained with great regularity since adopting the new technic). Since only a few of the cells are originally infected (on the average of about one in a hundred) there could be no marked increase in the percentage of infected cells unless the uninfected cells were outgrown by the infected cells. Table I, however, shows that approximately 40 per cent of the cells in mitosis were *Rickettsia*-free, while only 8.5 per cent of the cells not in mitosis were *Rickettsia*-free.

It therefore seems necessary to assume that *Rickettsiae* are set free from one cell and gain entrance to others. This must also occur *in vivo* when scrotal sac exudate is injected intraperitoneally.

In an attempt to obtain information on this point, we have cultivated normal tissues (striated muscle, lung, spleen and kidney) along with cultures of the scrotal sac exudate. In some cases the normal tissue has been in contact with the exudate originally and in others



the two tissues have been so placed that the growing cells from each would intermingle.

In the case of striated muscle which has been tested most thoroughly, we found heavy infection of what appeared to be sarcolemma cells in the late cultures (20 to 40 days *in vitro*) but no *Rickettsiae* were found in these cells in the earlier cultures. We have not been able entirely to rule out the possibility that these infected cells had grown around the muscle fibers from the exudate. The striated muscle cells do not grow but remain intact for 40 days or more. *Rickettsiae* were never found in the cytoplasm of these cells, even when they were in contact with heavily infected cells from the exudate for several weeks. Good growth of cells occurred in the cultures of lung, spleen and kidney.

TABLE I

	Degree of infection			
	Heavy	Medium	Light	Empty
Cells in mitosis	12 (9%)	16 (12%)	51 (39%)	52 (40%)
Cells not in mitosis	62 (28.5%)	75 (35%)	60 (28%)	18 (8.5%)

In the older cultures (21 to 40 days) an occasional infected cell was found along the edge of the normal tissue but we have never obtained a picture at all comparable to the unrestricted growth which occurred in the adjacent exudate.

We are unable to explain why the infection spreads and involves every cell descending from the exudate but does not (to any considerable extent) involve the cells descending from the normal tissues, in spite of prolonged contact between the two tissues. It seems possible that this fact may be explained on the basis of the experiments reported in Paper II. This work showed definitely that *Rickettsiae* did not multiply or even persist in phagocytic cells and that they did multiply voluminously in mesothelial cells. It suggested strongly that they were unable to multiply in fibroblasts. We have not been able to identify with certainty the cells present in our successful cul-

tures from the scrotal sac exudate. Phagocytic cells apparently do not persist and the cultures soon appear to be composed of cells morphologically consistent with either mesothelial cells or fibroblasts. Study of the explanted exudate fragments shows (aside from the macrophages and cells of the granulocytic and lymphatic series) only sheets of polygonal cells, closely fitted together and definitely resembling mesothelium rather than connective tissue. We believe therefore that our cultures as a rule are pure cultures of mesothelium and think that this may explain the extensive multiplication of *Rickettsiae* in them and the failure of *Rickettsiae* to grow in the cultures of normal tissues, which are presumably largely fibroblasts. This theory is strengthened by the fact that we have in several instances noted heavy infection of pleural or peritoneal lining cells covering normal lung and spleen in cultures of 20 to 25 days duration. This problem requires further study.

#### *Behavior of Rickettsiae in Dividing Cells*

Organisms are commonly found in cells undergoing mitotic division but are less numerous in such cells than in resting cells. This fact is brought out in Table I. Their behavior in dividing cells is interesting. They almost invariably assume a globular form and the cytoplasm around the dividing chromosomes is free from them. They usually become massed together along the cytoplasmic membrane, especially at the poles of the cells. Frequently they are present in clusters at each end of the cells, suggesting agglutination. Organisms in dividing cells are frequently stained blue but this is probably because of the lowered pH. These observations suggest that the dividing cell is an unfavorable medium for the organisms (probably because of its acidity) but it seems probable that a certain number of organisms usually survive the process and multiply in the daughter cells when conditions are restored to normal.

Typhus *Rickettsiae* have never been seen in nuclei, in spite of careful search. In heavily infected cells, the nucleus is often compressed or obscured, or, if visible, is recognized as a clear zone in the center of the dark purple mass of *Rickettsiae* in the cytoplasm of the cell. This fact is in contradistinction to the behavior of spotted fever *Rickettsiae* (4).

*Oxygen Requirements of Typhus Rickettsiae*

The voluminous multiplication of organisms described here has been obtained in cultures exposed to air within the Petri dishes. Whether oxygen gains entrance to the cells under such conditions in larger amounts than in living animals is problematical. The oxygen tension within living cells is generally believed to be practically zero.

The technic used for obtaining anaerobiosis in the tissue cultures was as follows:

The cultures were set out in Petri dishes in the usual way. They were then placed in a glass jar and the covers were partly raised to establish free communication between the atmosphere of the Petri dishes and that of the interior of the jar. The covers were held in this position by small pyramids of plasteline. The glass jar was then sealed with the aid of wax and a brass cover having a single outlet and stop-cock. Evaporation was kept at a minimum by covering the bottom of the jar with wet absorbent cotton. Anaerobiosis was obtained by evacuating and washing out three times with hydrogen gas in contact with heated platinized asbestos. A solution made by adding 1 drop of methylene blue to 5 cc. of broth without dextrose was placed in each jar as an indicator. Decolorization of this solution was always complete in 12 hours.

Several groups of cultures proven to be heavily infected after 16 to 36 days of growth in the usual way were put under the most perfect conditions of anaerobiosis at our command. The degree of anaerobiosis was checked by the decolorization of methylene blue. These cultures remained under anaerobic conditions for 8 to 12 days at 32°C. At the end of this time they were studied histologically and by inoculating guinea pigs. The majority of the inoculated animals reacted positively but with relatively long incubation periods (6 to 10 days). In four cultures studied histologically, relatively few intact cells and no cells of viable appearance were present. *Rickettsiae* of the globular or coccoid forms were numerous in many of the intact cells, but it was obvious that they were merely surviving under adverse conditions. No organisms were found in the fibrin clot at a distance from the tissue. *Rickettsiae* were only about one-twentieth as numerous as in the aerobic control cultures, in which the growth of cells continued.

This experiment shows that factors other than oxygen tension are responsible for the multiplication of *Rickettsiae* within the cells and their lack of multiplication in the fibrin clot. (The clot presumably

contains many of the chemical constituents of cytoplasm, since cells are continually disintegrating.)

### *Reaction of Medium*

Groups of cultures have been set out in which the pH of the medium was altered both on the acid and on the alkaline side. Sodium hydroxide and hydrochloric acid were added to the splenic extract in such amounts that the desired pH was effected when an equal volume of plasma was added. The pH of the final culture medium thus produced was determined colorimetrically. Moderate growth of cells was obtained at a pH of 6 and at a pH of 8. In both cases, no effect was noted on the number of *Rickettsiae* found in the section or on the infectivity. The cultures were incubated only for about 6 days. It seems doubtful if such changes in pH of the medium exert any marked effect on the intracellular pH so that these experiments probably are of slight value.

### *Relation of Typhus Rickettsiae to Metabolism of Host Cells*

Upon the addition of 1 drop of 1 per cent calcium chloride to cultures, growth of cells was, as far as could be determined, completely inhibited. After 17 days *in vitro*, however, many intact cells were present and the majority of cells appeared not to have undergone necrosis. Cultures of this group were as heavily infected as those in which the cells had multiplied.

In some instances, also, heavily infected cells were found in cultures grown for 2 weeks at 26°C. in which there had apparently been no growth of cells.

As mentioned above, heavily infected cultures became non-virulent and *Rickettsia*-free in 7 days in instances in which the cells did not survive transfer. Under anaerobic conditions which eventually cause death of the cells, there was a marked diminution in the number of *Rickettsiae* seen in sections and an increase in the incubation period of the reproduced infection, as compared with the controls.

It appears therefore that typhus *Rickettsiae* require for their propagation and survival, cells in which metabolism is taking place. They grow and persist in cells which are living and multiplying and in cells which are merely kept alive. When cells die, however, they appear

to remain visible only slightly longer than the degenerating cytoplasm and nucleus of the dying cells.

#### SUMMARY

In tissue cultures grown at 32°C., typhus *Rickettsiae* increase rapidly within the cytoplasm of infected cells up to about the 14th day. At this time practically every cell is infected and the majority of cells are distended with organisms.

This condition remains constant as long as successful cultures of the cells can be maintained (up to 52 days).

Loss in virulence does not take place during this period *in vitro*.

The number of *Rickettsia*-filled cells found in sections and the incubation period of the infection resulting from inoculation of cultures from each age group are definitely correlated.

The behavior of typhus *Rickettsiae* in dividing cells is described and methods of spread of the infection other than by mitosis of cells are discussed.

Normal tissues do not become infected *in vitro* to any considerable extent in spite of prolonged proximity to heavily infected cultures of scrotal sac exudate.

Complete anaerobiosis and alterations in pH do not alter the intracellular location of the organism in tissue cultures.

The organisms are not seen within nuclei of infected cells. They remain intact and infective for several weeks in cells which are kept alive but not multiplying. They disappear in less than 1 week, however, when the cells undergo degeneration.

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#### EXPLANATION OF PLATE 6

All illustrations are from paraffin sections of tissue cultures fixed in Regaud's fluid and stained by the Giemsa method.

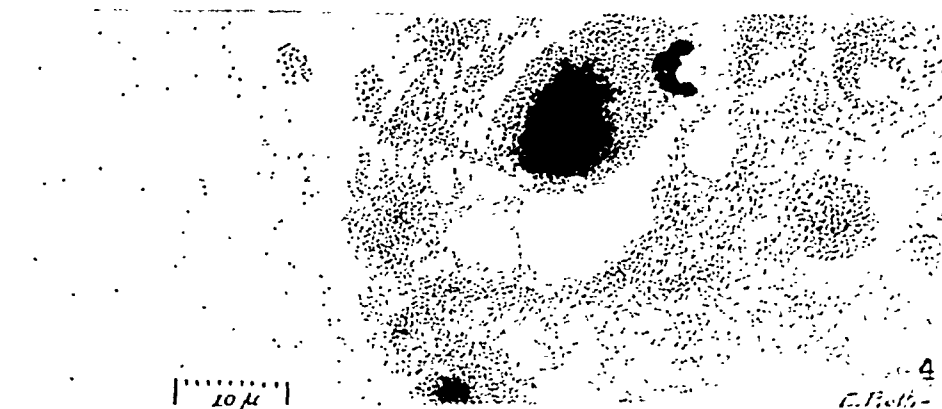
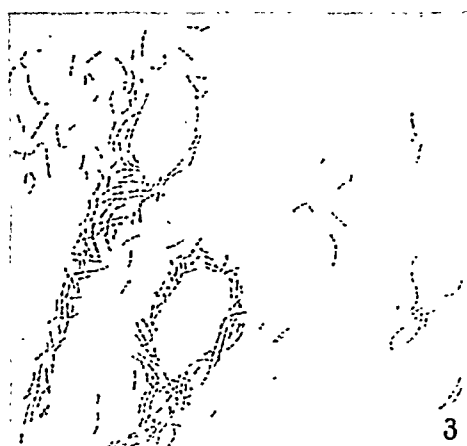
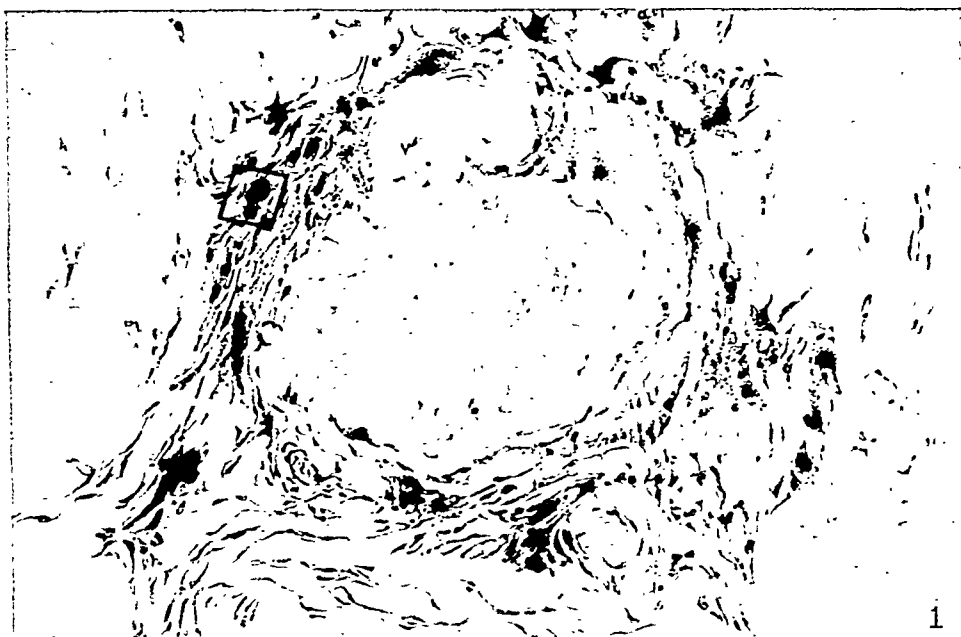
FIG. 1. Low power view of a tissue culture heavily infected with typhus *Rickettsiae*, on the 45th day *in vitro*. The dark structures (one of which is outlined) are colony-like masses of *Rickettsiae* within distended cells.  $\times 90$ .

FIG. 2. High magnification of the field within square outlined in black ink in Fig. 1. The individual organisms in the center of the distended cells cannot be resolved but definite organisms are seen peripherally.  $\times 1200$ .

FIG. 3. Young growing cells lightly infected with thread forms of *Rickettsiae*. From a culture 51 days old.  $\times 1200$ .

FIG. 4. From an infected culture on the 52nd day *in vitro*. The cells are largely disintegrated. *Rickettsiae* lie free in the plasma but are still concentrated in the remaining cytoplasm of intact cells. Organisms have diffused into the surrounding fibrin clot, but are present there in relatively small numbers and are evidently not multiplying there.  $\times 1200$ .





(Pinkerton and Hans: Typhus fever. IV)





## TYPHUS FEVER

### V. THE EFFECT OF TEMPERATURE ON THE MULTIPLICATION OF RICKETTSIA PROWAZEKI IN TISSUE CULTURE

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(Received for publication, March 29, 1932)

#### *Material and Methods*

Scrotal sac exudate from guinea pigs reacting to endemic typhus was explanted, using the technic previously described (1, 2). The cultures were incubated at 27°C., 32°C., 37.5°C. and 41°C. The incubators were not maintained accurately at these four temperature levels but variations were always less than 0.5° in either direction. As a rule eight individual cultures (all set out in the same Petri dish) were put to begin with in each of the four incubators. At various intervals the Petri dishes were opened, one or more cultures from each group fixed for histological study, one or more injected into a guinea pig and the remainder washed and reimplanted in fresh extract and plasma.

#### OBSERVATIONS

*Growth of the Cells.*—At 27° the cells usually rounded up and failed to grow. In about half of the cultures, however, there developed a distinct halo of rounded cells around the original tissue, probably largely the result of ameboid motion by the phagocytic cells. In two of the fifty-six cultures incubated at this temperature, however, there was fairly good growth of spindle-shaped cells and in one instance, mitotic figures were found in sections on the 11th day *in vitro*.

At 32° the cultures (with few exceptions) showed slow but very satisfactory growth. Cells grown at this temperature remained stationary but viable for long periods. One group of six cultures was left for 21 days without transfer. The cells did not round up or darken, and the clot was not liquified. At the end of this period they were washed and reimplanted and excellent growth occurred in all six.

At 37.5° the growth was uniformly excellent but more frequent transfers were necessary (usually at intervals of about 6 days).

At 41° growth was uniformly excellent and even more rapid than at 37.5°. Transfers at 6 day intervals seemed sufficient, however, to maintain the cells in good condition.

### *Growth of Rickettsiae*

In Table I, isolated results have been taken from five different experiments and tabulated with reference to the number of days *in vitro*. The cultures having the same number all belong to the same group. For example, Cultures 101-1, 101-2, 101-3 and 101-4, were all set out on the same day from the same material and differed only in the temperature at which they were incubated. The results are shown graphically in Chart 1. The criteria for infection of inoculated guinea pigs were (1) a febrile reaction, (2) scrotal swelling and redness with palpable adhesions in the scrotal sac, (3) exudate in the scrotal sac and (4) the presence of *Rickettsia*-filled cells in this exudate. If 1 and 2 were definitely present, the animal was killed and the positive reaction was confirmed by 3 and 4. Animals in which no fever or scrotal reaction occurred within 25 days, or in which only a slight and doubtful rise in temperature was observed without involvement of the scrotum or tunica were reinoculated with an adequate dose of fresh scrotal sac exudate, together with controls.

For tabulating the histological appearance of the cultures, an arbitrary system was adopted as follows:

0	No <i>Rickettsiae</i> found in $\frac{1}{2}$ hour search.
(+)	Rare infected cells.
+	1 to 5 infected cells per section.
+(+)	Intermediate between + and ++.
++	25 to 50 infected cells per section.
++(+)	Intermediate between ++ and +++.
+++	Infection of 50 per cent of the cells present.
+++(+)	Intermediate between +++ and ++++.
++++	Infection of practically every cell present.

The illustrations accompanying Paper IV (published simultaneously with this paper) show the appearance of the very heavily infected cultures (++++).

Table I and Chart 1 show that the organisms disappeared quite rapidly from cultures grown at 41° and 37.5°. At 26° more variable

TABLE I

*Multiplication of Rickettsia prowazeki in Tissue Cultures at Various Temperatures*

Temperature	No. of culture	Length of time in vitro	Growth of cells	<i>Rickettsiae</i> histologically	Guinea pig inoculation	Incubation period	Result of reinoculation
		days				days	
26°	97-1	4(0)*	0	(+)	Positive	13	Positive
	101-1	7(0)	Very slight	(+)	Positive	12	
	97-1	11(1)	Very slight	+	Positive	7	
	103-1	13(1)	0	(+)	Positive	12	
	101-1	14(1)	0	(+)	Positive	10	
	105-1	18(2)	0	0	Negative		
	101-1	21(1)	0	0	Negative		
32°	101-2	7(0)	Good	+	Positive	6	Positive
	97-2	11(1)	Good	++	Positive	5	
	103-2	13(1)	Good	+(+)	Positive	6	
	101-2	14(1)	Excellent	++(+)	Positive	4	
	105-2	18(2)	Excellent	+++	Positive	4	
	101-2	21(0)	Good	+++	Positive	2½	
	108-2	28	Excellent	+++(+)	Positive	4	
	101-2	36(1)	Good	+++(+)	Positive	3	
	101-2	51(2)	Excellent	+++(+)	Positive	3	
37.5°	97-3	4(0)	Excellent	+(+)	Positive	6	Positive
	101-3	7(0)	Excellent	(+)	Positive	10	
	97-3	11(1)	Good	0	Negative		
	103-1	13(1)	Good	0	Negative		
	101-3	14(1)	Excellent	0	Negative		
	105-3	18(2)	Excellent	0	Negative		
41°	108-4	1	Good	(+)	Positive	9	Positive
	108-4	2	Excellent	0	Positive	16	
	108-4	3	Excellent	0	Negative		
	97-4	4(0)	Good	0	Negative		
	105-4	4(0)	Excellent	0	Negative		
	103-4	5(0)	Excellent	0	Negative		
	101-4	7(0)	Excellent	0	Negative		
	97-4	11(1)	Excellent	0	Negative		
	103-4	12(1)	Excellent	0	Negative		
	101-4	14(1)	Excellent	0	Negative		
	105-4	18(2)	Excellent	0	Negative		

\* Figures in parentheses refer to the number of times the cultures were transferred during the growth period.

results were obtained, but organisms did not multiply extensively, and disappeared entirely on or before the 18th day. At 32° practically every cell became infected and this condition was apparently maintained as long as the cells were kept alive. Cultures from Group 101 which were actively growing and heavily infected on the 51st day, did not survive transfer. On the day after transfer (the 52nd day *in vitro*) the cells had rounded up and were obviously dying. Cultures of this group on the 59th and 66th days, were found to be histologically *Rickettsiae*-free and inoculated animals did not react.

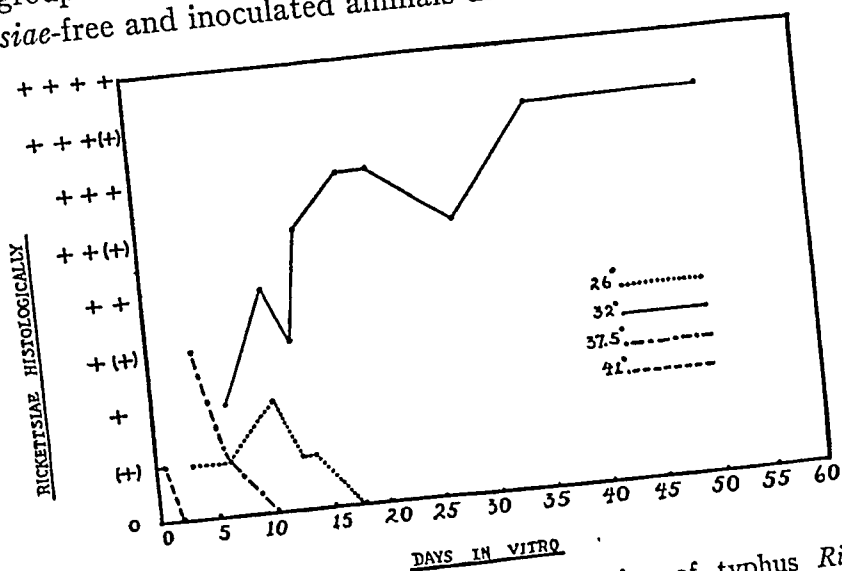


CHART 1. Effect of temperature on multiplication of typhus *Rickettsiae* *in vitro*.

### DISCUSSION

One of the questions which we thought might be answered by studying typhus infection in tissue cultures was whether or not the cells, isolated from the body and kept alive and growing under artificial conditions, would be capable of acquiring immunity to the infection. From the above results it appears that immunity is acquired rapidly at 41°C. (in 3 days) and more slowly at 37.5°C., while at 32°C. immunity does not develop. (The results at 26°C. cannot be included in this problem, since the cells do not survive or multiply to any considerable extent. The irregular positive results obtained at this temperature are probably due to the maintenance of metabolism in a few cells for a considerable period of time.)

It is, of course, possible that the inhibitory effects of temperature are exerted directly on the organisms. This seems improbable, however, in view of the fact that *Rickettsiae* multiply luxuriantly in the scrotal sac of inoculated guinea pigs where the temperature is often above 37.5°C. Furthermore, if the effect of temperature were a direct one, one would not expect, at 37.5°, to find an initial multiplication, followed by complete disappearance.

#### SUMMARY AND CONCLUSIONS

The temperature at which tissue cultures infected with typhus *Rickettsiae* are incubated has been shown to exert a marked influence on the intracellular multiplication of *Rickettsia prowazeki*.

At 41°C. the organisms were not found in the cultures histologically on and after the 2nd day *in vitro*, and the cultures were non-virulent on and after the 3rd day *in vitro*, in spite of good preservation and growth of the cells.

At 37.5°C. organisms were absent from the cultures histologically and the cultures were non-virulent on and after the 11th day *in vitro*, in spite of good preservation and growth of the cells.

At 32°C. good but slow growth of cells occurred and organisms were found in increasing numbers histologically up to about the 21st day *in vitro*. At this time, 50 to 99 per cent (approximately) of the cytoplasmic volume of the cultures was occupied by *Rickettsiae*. From the 21st day to the 51st day (the limit to which cultures have been carried successfully) this condition of unrestricted multiplication remained practically unchanged. Inoculation of guinea pigs with single cultures after varying lengths of time *in vitro*, (up to the 51st day) always resulted in reproduction of typhus in a characteristic manner.

At 27° the cell growth was negligible, but many cells remained alive for 10 days or more. Organisms were absent from the cultures histologically and the cultures were non-virulent on and after the 18th day *in vitro*.

The only histological preparations showing unrestricted multiplication of the organisms (infection of the majority of the cells present) were of cultures incubated at 32°C.

It is believed that the detrimental effect of the higher temperatures

(37.5° and 41°C.) on the multiplication of the organism is exerted indirectly, by stimulation of the defence mechanism of the cells.

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# SPOTTED FEVER

## I. INTRANUCLEAR RICKETTSIAE IN SPOTTED FEVER STUDIED IN TISSUE CULTURE

BY HENRY PINKERTON, M.D., AND G. M. HASS, M.D.  
(From the Department of Pathology, Harvard Medical School, Boston)

PLATE 7

The object of this paper is to describe an unusual morphological picture obtained by studying spotted fever infection by the tissue culture method.

Wolbach and Schlesinger (1) in 1923 cultivated tissues infected with *Dermacentor xenus rickettsi*. They maintained infectivity *in vitro* for 28 days and found the specific organism in increasing numbers, histologically, up to the 14th day. Their results indicated that definite multiplication of the organism had taken place, but the number of infected cells found histologically was relatively small.

In view of the voluminous multiplication of typhus *Rickettsiae* which we have obtained recently in tissue culture (2, 3), it seemed desirable to restudy spotted fever by the new technic.

The strain of spotted fever used in the experiments to be reported here was an atypical one, isolated in Minneapolis, Minnesota. We are indebted to Dr. H. A. Reimann for the opportunity of studying it. A description of the strain, including immunological evidence that it belongs in the spotted fever group, has been published by Reimann, Ulrich and Fisher (4).

This strain was chosen for tissue culture work because of the fact that guinea pigs reacting to it develop an acute inflammation of the scrotal sac, often resulting in a membranous exudate similar to that found in endemic typhus. Cells infected with *Rickettsiae* are present in relatively small numbers in this exudate in the Reimann strain but are probably more numerous than in fragments of infected testis and cremasteric muscle in typical Rocky Mountain spotted fever.



the latter disease no exudate is present in the scrotal sac and one must rely upon the presence of *Rickettsiae* in the vascular lesions in the fragments of tissue used as explant material. Another advantage of this strain is that the presence of *Rickettsiae* can be easily confirmed by making smears of the exudate so that the most desirable material for setting out cultures may be selected.

### *Material and Methods*

Membraneous exudate from the scrotal sac on the 2nd or 3rd day of fever was floated in Tyrode's solution, cut into small fragments and explanted, using the technic previously described for similar experiments with typhus *Rickettsiae* (2, 3). The cultures were incubated at 32°<sup>1</sup> and transferred usually every 6 or 7 days. The cultures were carried on for varying lengths of time ranging up to 32 days. At intervals certain cultures of each group were fixed and stained by the technic previously described and others were injected into guinea pigs for virulence tests. The cultures for injection, together with the fibrin clot in which they were embedded, were picked up with forceps and floated in 4 cc. of Tyrode's solution, taken up into a syringe with a needle of large calibre and injected intraperitoneally. They were then taken up into a syringe with a needle of large calibre and injected intraperitoneally, taking care in each case that the culture did not adhere to the sides of the syringe. (By holding the syringe against a dark background during injection it was possible to see the culture disappear through the proximal end of the needle.) The cultures for histologic study were fixed in Regaud's fluid and embedded in paraffin. Serial sections about 7 micra in thickness were stained by the Giemsa method.

### RESULTS

The guinea pigs injected with cultures of various ages all reacted positively. It was noteworthy that a marked scrotal sac inflammation, resulting in palpable adhesions between visceral and parietal layers, occurred constantly in animals inoculated from the tissue cultures, whereas it was relatively rare in animals inoculated in the usual way with blood or scrotal sac exudate. This we ascribe to the higher concentration of *Rickettsiae* in the tissue cultures.

Large numbers of infected cells were found in paraffin sections of the cultures, and excellent morphological pictures of the intracellular parasites were obtained. In several cultures, more than half of the

<sup>1</sup> In a recent publication (5) it is shown that the temperature of incubation has a marked influence on the multiplication of typhus *Rickettsiae* in tissue culture.

cells present contained *Rickettsiae*. The organisms (as in the case of typhus) were confined to the interior of cells and never seen in the plasma clot except in very rare instances when cells had ruptured. They were never seen in the clot at a distance from the growing tissue.

The outstanding feature of the picture was the apparent predilection of these spotted fever *Rickettsiae* for the nuclei of the cells. In several preparations, the majority of infected cells showed only nuclear involvement. Infection of the cytoplasm without nuclear involvement was fairly common in all preparations, however. Cells in which both nuclei and cytoplasm were infected were quite rare. Within the nuclei the organisms were closely packed while in the cytoplasm they were almost invariably diffusely scattered. The majority of cells in which the cytoplasm was infected contained less than fifty *Rickettsiae*, and in no instance did the entire cytoplasmic volume become occupied by closely packed *Rickettsiae*.

Morphologically, the spotted fever *Rickettsiae* described here show great pleomorphism. The organisms range from minute coccoid bodies, about  $0.20\ \mu$  in diameter to long thread forms (mostly chains of bacilli) up to  $0.8\ \mu$  in diameter and  $15\ \mu$  in length. The thread forms were much more frequent in the cytoplasm and the smaller bacillary and coccoid forms in the nucleus. Rarely, however, intranuclear rods ranging up to  $4\ \mu$  in length were seen.

The organisms varied in their staining reaction from blue through the purples to red, depending on the degree of differentiation, but never attained quite the bright red color of the granules of polymorphonuclear leucocytes. In the cytoplasm they appear in some instances to retain the blue somewhat longer in the process of differentiation than did the fibrin clot in which the cells were embedded but the intranuclear forms were more markedly eosinophilic.

The various stages of intranuclear infection are shown in the accompanying illustration (Fig. 1). The initial stage is the appearance of from one to eight or more (most frequently one to three) small spherical groups of closely packed *Rickettsiae*, purplish red to bright red in color, and standing out clearly from the blue substance of the nucleus, even in undifferentiated sections. These clusters were composed of from three or four to fifty or more individual organisms. In the

the latter disease no exudate is present in the scrotal sac and one must rely upon the presence of *Rickettsiae* in the vascular lesions in the fragments of tissue used as explant material. Another advantage of this strain is that the presence of *Rickettsiae* can be easily confirmed by making smears of the exudate so that the most desirable material for setting out cultures may be selected.

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The organisms varied in their staining reaction from blue through the purples to red, depending on the degree of differentiation, but never attained quite the bright red color of the granules of polymorphonuclear leucocytes. In the cytoplasm they appear in some instances to retain the blue somewhat longer in the process of differentiation than did the fibrin clot in which the cells were embedded but the intranuclear forms were more markedly eosinophilic.

The various stages of intranuclear infection are shown in the accompanying illustration (Fig. 1). The initial stage is the appearance of from one to eight or more (most frequently one to three) small spherical groups of closely packed *Rickettsiae*, purplish red to bright red in color, and standing out clearly from the blue substance of the nucleus, even in undifferentiated sections. These clusters were composed of from three or four to fifty or more individual organisms. In the

smaller clusters, the rod-shaped organisms frequently formed a peripheral ring. A definite halo was always present about clusters of all sizes. In some instances the individual organisms of a group could not be resolved and the clusters appeared as hyaline masses (see upper left cell in Fig. 1). In the later stages, the clusters apparently become confluent and may occupy more than nine-tenths of the volume of the nucleus. There was invariably a pale clear ring at the periphery of the nucleus, however.

The nucleus itself was in some instances considerably swollen and often definitely wrinkled. In several cells the nuclear membrane appeared to have broken down so that nucleoplasm and cytoplasm had fused. In degenerating cells, clusters of *Rickettsiae* appeared as irregularly granular reddish purple structures, which, without study of intermediate stages, would not be accepted as microorganisms (see lower right cell in Fig. 1).

#### COMMENT

Wolbach (6) in 1919 found the specific organism of Rocky Mountain spotted fever massed within nuclei of epithelial cells in the gut, hypoderm and salivary gland of the tick. In spite of careful study by methods apparently adequate for its demonstration, it has never before been found in an intranuclear position in the tissues of any of its mammalian hosts. Its localization in nuclei in cultures of guinea pig tissues is therefore of considerable interest. The difference between the *in vivo* and *in vitro* pictures may depend on the lower temperature at which the tissue cultures were incubated. This question is being studied. We are also studying other strains of spotted fever by the same technic, since it seems improbable that the lesions described here are peculiar to the somewhat atypical strain used for this investigation.

The reason for the clustering of the organisms in nuclei and their diffuse distribution in the cytoplasm of cells is not apparent. Typhus *Rickettsiae* occasionally form separate groups in the cytoplasm of the cells in which they grow but these groups are rarely sharply outlined. The nucleus is less fluid than the cytoplasm and it seems possible that the aggregations of organisms may be comparable to bacterial colonies in solid artificial media.

The contrast between the behavior of spotted fever and typhus *Rickettsiae* in tissue culture is striking. The majority of cells infected with typhus *Rickettsiae* are filled almost to the point of bursting, but it is only the cytoplasm which is involved. In sections of such distended cells, the nucleus often stands out as a clear zone in the center of a dark purple mass of organisms. In spotted fever, it is the nucleus which becomes packed with organisms while the cytoplasm is only sparsely infected. Furthermore, spotted fever *Rickettsiae* are found within phagocytic cells (identified in the culture by their content of hemosiderin) while typhus *Rickettsiae* are strictly confined to non-phagocytic cells (7, 2).

Morphologically, the two organisms appear very similar when studied by this method. In size and shape they are practically identical. The spotted fever organisms in the cytoplasm are more frequently stained blue than the typhus organisms, but this is not true of the intranuclear forms.

It seems necessary to comment on the resemblance of the intranuclear clusters of *Rickettsiae*, especially when imperfectly fixed and stained, to certain of the structures of unknown nature found within cells in the so called virus diseases. The concept that some of these structures are masses of organisms has been expressed from time to time and in recent years has been especially sponsored by Goodpasture (8). Cowdry and Kitchen (9), however, on the basis of the lack of iron and thymonucleic acid and the intense acidophilic properties of the inclusion bodies believe that they are the result of chemical and physical changes.

The etiology of spotted fever has been thoroughly cleared up and this disease has been definitely taken out of the heterogeneous group of virus diseases. It is similar to virus diseases, however, in the definite association of infectivity with cells rather than with body fluids, and in the fact that the etiologic agent has not been made to multiply in artificial media in the absence of living cells.

The lesion described here is the first instance in which a definite microorganism has been shown to be parasitic in clusters in the nuclei of mammalian tissues. It therefore seems not improbable that some of the unresolved intranuclear structures now classed as inclusion bodies may be of a similar nature.

## SUMMARY

Spotted fever infection has been studied in tissue cultures grown at 32°C. The behavior of spotted fever *Rickettsiae* is compared and contrasted with that of typhus *Rickettsiae* under similar conditions. The spotted fever organisms multiply extensively in the nuclei of cells where they form spherical clusters of various sizes. These structures are compared and contrasted with intranuclear inclusion bodies.

*Note.*—After preparing this paper for publication, similar results were obtained from the strain of "Eastern spotted fever" isolated by Rumreich, Dyer and Badger (10). Cultures from guinea pigs reacting to this strain were set out both from the scrotal sac and from the spleen. In both cases the cultures became heavily infected with *Rickettsiae* on the 11th and 18th days and the organisms were massed within nuclei in this strain also. This is evidence of the essential similarity of the two strains, and additional evidence that both belong in the spotted fever group.

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## EXPLANATION OF PLATE 7

FIG. 1. All cells are from a single paraffin section of a tissue culture of infected scrotal sac exudate from a guinea pig reacting to spotted fever. Five of the cells are from a single field, while the others have been selected from other parts of the section to show more completely the morphological range. The ratio of cytoplasmic to nuclear infection is about the same as that in the section as a whole. The cultures were fixed in Regaud's fluid and embedded in paraffin. Serial sections about 7 micra in thickness were stained by the Giemsa method.  $\times 1500$ .

Note especially the cells in the upper left and lower right corners, in which individual organisms cannot be resolved. The resemblance of the structures in these cells to inclusion bodies is striking.

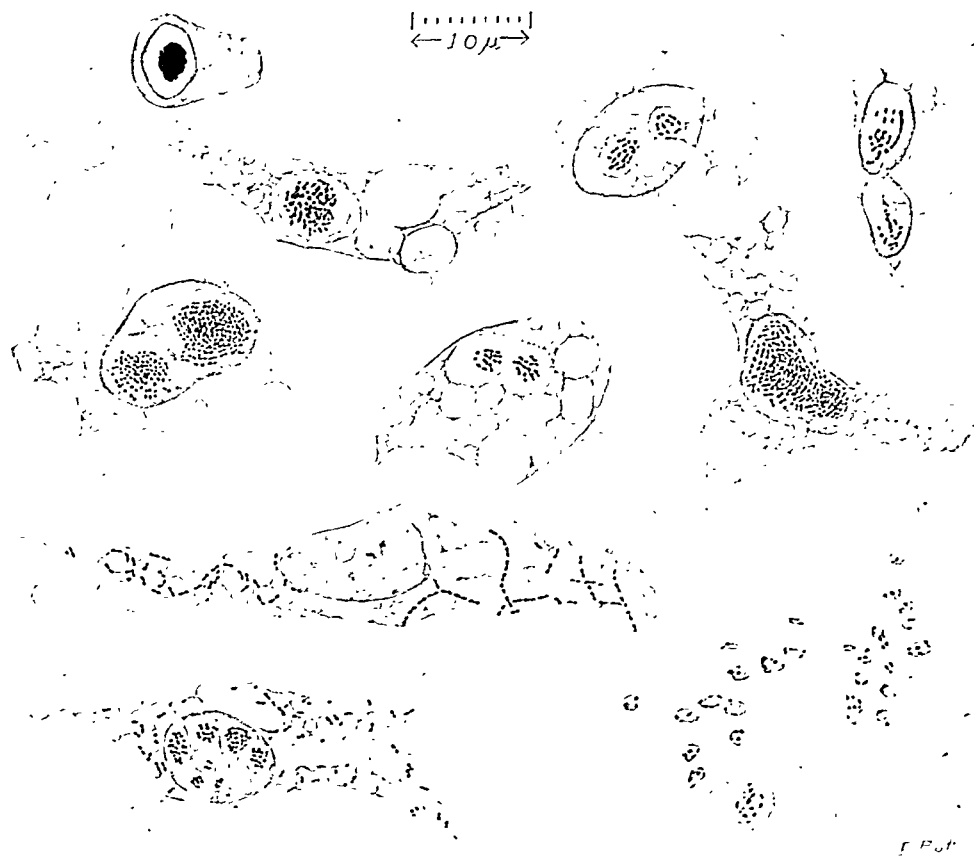


FIG. 1

(Pinkerton and Hass: Spotted fever. 1)



sisting of material brightly stained with eosin in which degenerated leucocytes are found (Fig. 6, Experiment 9, Table I). A section of the omentum when graphite has been injected intraperitoneally about 18 hours after the onset of the inflammatory reaction shows the bulk of this material distributed within the strands of a coarse fibrinous network (Fig. 7, Experiment 9, Table I); there is no sign of graphite in the thrombus occluding the retrosternal lymphatic (Fig. 6).

3. When concentrated urea is injected into the peritoneal cavity either simultaneously with or following an inflammatory irritant, the lumina of the retrosternal lymphatics appear either unoccluded (Fig. 2, Experiment 3, Table I), or only partially obstructed by fibrinous strands (Fig. 5, Experiment 12, Table I). Frequently a network of fibrin is seen at the periphery of the lumen, the central area of the latter being left clear. Although relatively few leucocytes are seen within the delicate reticulum, graphite particles often appear in its meshes.

Sections through the omentum of animals that have received aleuronat and urea intraperitoneally usually reveal the presence of pronounced congestion, capillary dilatation, and some areas of extravasated red cells but in contrast to the findings in animals that have been treated with aleuronat and distilled water, there is no marked fibrinous network. Only occasionally are a few fragments of fibrin seen within tissues infiltrated with polymorphonuclear leucocytes.

A comparison of the above histological picture with the results obtained on the dissemination of graphite to the retrosternal lymph nodes, as listed in Table I, shows an interesting correlation. When the particulate material is injected into a normal peritoneal cavity it accumulates to a very large extent in the nodes (+++), and the lumen of the afferent retrosternal lymphatic remains unobstructed (see Fig. 1, Experiment 5, Table I).

When graphite is introduced into a peritoneal cavity previously treated with aleuronat and distilled water, none of this material penetrates to the lymph nodes (0), and the lumen of the retrosternal lymphatic is completely occluded by a thrombus characterized by many leucocytes within a delicate fibrinous network (see Figs. 3, 4, and 6, Experiments 5 and 9, Table I).

However when graphite particles are injected into a peritoneal

cavity that has been treated with aleuronat and concentrated urea the retrosternal lymph nodes are either intensely loaded with carbon (+++), or else this substance is present in only moderate amounts (+ or trace). When graphite is abundant in the nodes (+++), the lumen of the afferent lymphatic vessel correspondingly is unobstructed (see Fig. 2, Experiment 3, Table I). On the other hand, when the particulate material is found in the nodes in relatively smaller amounts (+ or trace), the lumen of the retrosternal lymphatics is partially blocked by a fine reticulum (see Fig. 5, Experiment 12, Table I).

The above facts, coupled with the *in vitro* studies described at the beginning of this paper, lead to the conclusion that concentrated urea inhibits either wholly or in part the fixation of particulate matter at the site of inflammation by its tendency to prevent the formation of a fibrin network and of thrombi in the tributary lymphatics.

#### *The Inhibitory Effect of Concentrated Urea Solution on the Fixation of Iron at the Site of Inflammation*

The results of the above experiments were substantiated by the use of another testing material, ferric chloride. In an earlier publication (3) it had been shown that this iron salt injected into an inflamed area was fixed *in situ* by the inflammatory reaction.

An acute inflammatory reaction was induced in the peritoneal cavity of rabbits by the introduction of 10 cc. of an aleuronat suspension. This was immediately followed by the injection of 10 cc. of urea in a concentration of either 30 or 50 per cent. As control a second rabbit received an intraperitoneal injection of aleuronat followed by distilled water. After a variable interval of time 5 cc. of 0.25 per cent ferric chloride was injected intraperitoneally in each of the above animals and also into a third normal rabbit. When the animals were killed the retrosternal lymphatics and nodes were treated with acidified potassium ferrocyanide solution (Prussian blue reaction).

From Table II it is clear that the addition of concentrated urea to aleuronat inhibits the fixation of the iron salt at the site of inflammation.

#### *Fixation of Graphite Particles When Urea Injection Follows the Onset of Inflammation in the Peritoneal Cavity*

In some instances a concentrated urea solution was injected into the peritoneal cavity some time after the development of the inflam-

an area of inflammation treated with concentrated urea should allow the penetration of a dye injected at its periphery.

On the shaved abdominal skin of a white rabbit intracutaneous injections were performed in two distinct areas as follows: (1) 0.4 cc. of 50 per cent urea followed by 0.2 cc. of an infusion broth suspension of *Staphylococcus aureus*; (2) 0.4 cc. of distilled water followed by 0.2 cc. of the same bacterial suspension. After a variable interval of time 0.8 cc. to 1 cc. of 1 per cent trypan blue was injected in several points around each inflamed area and also at the periphery of a normal skin area. After several hours the areas were examined for the presence of dye. In two of the experiments the areas were treated with concentrated urea alone.

TABLE IV

*Penetration of Trypan Blue into Inflamed Cutaneous Area Treated with Urea When Dye Is Injected at Its Periphery*

Experiment No.	Interval between injection of irritant and that of dye	Total duration of inflammation	Penetration of dye into area treated with		
			Urea alone or urea followed by <i>Staph. aureus</i>	Distilled H <sub>2</sub> O followed by <i>Staph. aureus</i>	Normal area
	hrs. : min.	hrs. : min.			
1*	2 : 25	4 : 38	Trace		Trace
2*	2 : 32	4 : 34	+		+
3	3 : 35	7 : 15	+++	Faint trace	+++
4	3 : 56	20 : 00	+++	0	++
5	5 : 00	6 : 52	++	0	+
6	5 : 30	6 : 30	+	0	+
7	5 : 35	13 : 40	+++	0	+

\* Urea used as sole irritant. In all other experiments urea injection immediately followed by that of broth suspension of *Staph. aureus*.

The results presented in Table IV show that trypan blue failed to penetrate into areas that had received distilled water and bacteria. When, however, the inflammation had been induced by concentrated urea alone or combined with bacteria, penetration of the dye into the inflamed area readily occurred. These experiments extend the foregoing observations on the rôle of concentrated urea in preventing fixation at the site of inflammation.

#### *Concentrated Urea as an Inflammatory Irritant*

Experiments were set up to determine the effect of concentrated urea *per se* when injected into normal tissues.

When 50 per cent urea in small amounts (0.2 to 0.5 cc.) is introduced into the skin of a rabbit, the area immediately becomes congested in contrast to the initial blanching that occurs when, for instance, a suspension of *Staphylococcus aureus* is injected into the dermis. After a relatively short interval of time the urea-treated area becomes edematous and pin-point hemorrhages are frequently seen on its surface. In the earlier stage hemorrhagic congestion is the striking feature of the reaction, whereas in the later stages (24 hours or over) the area exhibits typical hemorrhagic necrosis. Histological examination in the acute stage shows a great deal of edema with pronounced capillary congestion and dilatation. Some capillaries display an interrupted endothelial wall with considerable extravasation of blood into the surrounding tissues. Fibrinous deposits are as a rule absent. In later stages polymorphonuclears and mononuclear phagocytes are found in abundance.

Concentrated urea is in itself a severe inflammatory irritant rapidly injuring the capillary wall and allowing the escape of plasma and red cells into the extracapillary spaces. Immediately after the introduction of various high concentrations of urea into the skin, intravenous injection of trypan blue is rapidly followed by the accumulation of this dye in the urea-treated areas. A typical experiment is described in the following protocol.

*Rabbit 5-67.*—At 11:40 a.m. 0.25 cc. of various concentrations of urea was injected intracutaneously in several areas of the abdominal and thoracic surfaces. The actual concentrations of urea injected were 50, 25, 20, 15, 10, 5, 1, 0.5 per cent, and finally distilled water. At 11:45 a.m. 10 cc. of 1 per cent trypan blue in saline was injected intravenously. At 11:49 a.m. areas injected with concentrations of urea ranging downwards from 50 per cent to 20 per cent were congested, whereas the areas with very low concentrations of urea and with distilled water

*Accumulation of Trypan Blue in Cutaneous Areas Treated with Urea*

Interval between intravenous injection of trypan blue and accumulation of dye in urea-treated areas <i>min.</i>	Concentration of urea injected, <i>per cent</i>								Distilled H <sub>2</sub> O
	50	25	20	15	10	5	1	0.5	
2	+	0	0	0	0	0	0	0	0
4	++	Trace	Faint trace	0	0	0	0	0	0
12	+++	+	Trace	0	0	0	0	0	0
33	+++	+	+	Faint trace	0	0	0	0	0
65	+++	++	++	+	0	0	0	0	0

an area of inflammation treated with concentrated urea should allow the penetration of a dye injected at its periphery.

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3	3 : 35	7 : 15	+++	Faint trace	+++
4	3 : 56	20 : 00	+++	0	++
5	5 : 00	6 : 52	++	0	+
6	5 : 30	6 : 30	+	0	+
7	5 : 35	13 : 40	+++	0	+

\* Urea used as sole irritant. In all other experiments urea injection immediately followed by that of broth suspension of *Staph. aureus*.

The results presented in Table IV show that trypan blue failed to penetrate into areas that had received distilled water and bacteria. When, however, the inflammation had been induced by concentrated urea alone or combined with bacteria, penetration of the dye into the inflamed area readily occurred. These experiments extend the foregoing observations on the rôle of concentrated urea in preventing fixation at the site of inflammation.

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*Accumulation of Trypan Blue in Cutaneous Areas Treated with Urea*

Interval between intra- venous injection of trypan blue and accu- mulation of dye in urea-treated areas  min.	Concentration of urea injected, per cent								Distilled H <sub>2</sub> O
	50	25	20	15	10	5	1	0.5	
2	+	0	0	0	0	0	0	0	0
4	++	Trace	Faint trace	0	0	0	0	0	0
12	+++	+	Trace	0	0	0	0	0	0
33	+++	+	+	Faint trace	0	0	0	0	0
65	+++	++	++	+	0	0	0	0	0

appeared blanched. Furthermore the dye had accumulated in the peripheral portions of the four congested areas containing the higher concentrations of urea. The relation between the concentration of urea used and the rapidity and degree of accumulation of the dye into the various skin areas is indicated by the preceding tabulation.

In order to ascertain if possible the mode of action of a concentrated urea solution on the capillary wall several experiments were performed on the web of the pithed frog. Less than 10 seconds after the application of 50 per cent urea to the web the capillary circulation ceased and the vessels became packed with red cells. The rapidity of this effect is especially striking since only after several minutes did an irritant as highly potent as croton oil produce a definite change in the capillary circulation. It remains to be seen whether the effect of concentrated urea on the capillary wall is related to the protein-solvent action of this substance or whether it is a type reaction accompanying a powerful inflammatory irritant.

Experiments were performed to determine the effect of a concentrated urea solution by itself on the fixation of graphite particles at the site of inflammation.

10 cc. of 50 per cent urea was injected into the peritoneal cavity of a rabbit. This was followed several hours later by 3.5 cc. of diluted graphite ink. After a variable interval of time the animal was killed with ether. Examination of the retrosternal nodes revealed extensive deposits of carbon. The results are listed in the following table.

Rabbit No.	Interval between injection of urea and that of graphite	Total duration of inflammation	Presence of graphite in retrosternal lymph nodes
	<i>hrs. : min.</i>	<i>hrs. : min.</i>	
3-68	5 : 14	6 : 32	+++
3-72	16 : 58	19 : 26	+++

Evidently graphite particles injected into an area of inflammation induced by concentrated urea alone disseminate readily to the tributary lymphatic nodes.

Histological examination of the retrosternal lymphatics revealed essentially no occlusion. A relatively narrow layer of fibrinous reticulum loaded with graphite particles within its strands could be seen in the peripheral portion of the lymphatic lumen as shown in

Fig. 8. Most of the lumen however is unobstructed and this can account for the free dissemination of a large amount of graphite to the lymph nodes. Concentrated urea usually produces intense congestion in the vessels of the peritoneal lining, with extravasation of large quantities of blood. Numerous red blood corpuscles and leucocytes can be seen in the lumen of the retrosternal lymphatics (Fig. 8).

#### DISCUSSION

As pointed out in previous publications (7-9) the initial injury of an irritant to the capillary wall by increasing its permeability allows the passage of fibrinogen (and other plasma proteins) into the extracapillary spaces. The early formation of a fibrinous network and of thrombi in lymphatics limits the spread of the irritant. The solvent action *in vitro* of urea on fibrin, and the inhibition of fixation by its means, coupled with the histological evidence of lymphatic blocking, furnish additional evidence of the importance of lymphatic blocking.

That the mechanical obstruction which localizes the irritant occurs very early in the development of the inflammatory reaction is shown by the fact that fixation of trypan blue was obtained in some experiments 30 minutes after the subcutaneous injection of an inflammatory irritant (1). When the irritant was injected into the peritoneal cavity, fixation of graphite particles could be demonstrated as early as 2 hours and 46 minutes after the introduction of the irritant. Further evidence on this point was obtained by microscopic examination of lymphatics draining an inflamed area of relatively short duration (7 hours). As illustrated in Fig. 4, graphite particles that have failed to reach the tributary lymph nodes when injected into an inflamed area are caught in a fibrinous reticulum within an afferent lymphatic. There is practically no phagocytosis of graphite granules in spite of the abundance of leucocytes present before the irritant at the onset of the inflammatory reaction which fixes the leucocytes have had time to perform effectively their phagocytic function. Later, however, phagocytes, doubtless reinforced in their activity by the opsonizing elements contained in serum, accomplish the final removal of the foreign substance. Since it has been demonstrated by the writer (4) that foreign proteins accumulate from the circulating blood stream into an inflamed



area and are fixed there, it is quite probable that in the case of specific inflammation circulating antibodies may be concentrated at the site of inflammation thus reinforcing the action of the leucocytes in disposing of the irritant.

Early fixation serves as the forerunner of the events whereby disposal of the foreign material occurs at the site of inflammation. By circumscribing the irritant it gives an interval of time for the leucocytes to assemble for the purpose of phagocytosis. The initial barrier caused by thrombosed lymphatics and coagulated serum in the tissue spaces of the inflamed area thus plays a definite rôle in immunity protecting the essential organs at the expense of local injury.

#### CONCLUSIONS

A concentrated urea solution effectively dissolves fibrin.

The injection into the peritoneal cavity of a urea solution (30 or 50 per cent) together with or after an inflammatory irritant (aleurone or ferric chloride) prevents wholly or in part the local fixation of graphite particles introduced subsequently. The histologic picture of the retrosternal lymphatics explains how this comes about. When free dissemination of graphite to the retrosternal nodes occurs, the lumen of the lymphatic vessel is unobstructed, whereas partial dissemination is accompanied by small fibrinous thrombi occluding the lumen in part only.

Trypan blue injected at the periphery of an inflamed skin area and treated with a concentrated urea solution and bacteria (*Staph. aureus*) penetrates readily into the area, whereas it fails to do so when introduced around an inflamed area consequent on the injection of distilled water and bacteria (*Staph. aureus*).

Concentrated urea *per se* is an inflammatory irritant. Graphite particles injected into a peritoneal cavity previously treated with concentrated urea penetrate freely to the retrosternal lymphatic nodes; the lymphatic vessel is relatively unobstructed. Trypan blue injected into the circulating blood accumulates rapidly in cutaneous areas almost immediately after the latter have been treated with concentrations of urea ranging from 50 per cent down to 20 per cent.

The results of this study furnish evidence, in addition to that already provided, that fixation of foreign substances is primarily due

mechanical obstruction caused by a fibrin network and by thrombosed lymphatics at the site of inflammation. The significance of fixation in relation to immunity and its bearing upon some of the other processes involved in the inflammatory reaction have been stressed.

My thanks are due to Dr. Granville A. Bennett for taking the photographs, and to Mrs. Miriam F. Menkin for assistance in the preparation of this paper.

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## EXPLANATION OF PLATES

## PLATE 8

- FIG. 1. Retrosternal lymphatic vessel draining normal peritoneal cavity of control rabbit (Experiment 5, Table I). Graphite particles injected into the peritoneal cavity disseminated freely to the retrosternal lymphatic nodes (+++).  $\times 189$ .
- FIG. 2. Retrosternal lymphatic vessel draining peritoneal cavity 6 hours and 30 minutes after 20 cc. aleuronat suspension in 50 per cent urea had been introduced into it (Experiment 3, Table I). Graphite particles injected into the peritoneal cavity disseminated freely to the retrosternal lymphatic nodes (+++).  $\times 89$ .
- FIG. 3. Retrosternal lymphatic vessel draining peritoneal cavity about 7 hours after it had been treated with 10 cc. of aleuronat suspension followed by 10 cc. of distilled  $H_2O$  (Experiment 5, Table I). The lumen is occluded by a fibrinous thrombus containing leucocytes in abundance. Graphite particles injected into the peritoneal cavity failed to disseminate to the retrosternal lymphatic nodes (0).  $\times 81$ .

area and are fixed there, it is quite probable that in the case of specific inflammation circulating antibodies may be concentrated at the site of inflammation thus reinforcing the action of the leucocytes in disposing of the irritant.

Early fixation serves as the forerunner of the events whereby disposal of the foreign material occurs at the site of inflammation. By circumscribing the irritant it gives an interval of time for the leucocytes to assemble for the purpose of phagocytosis. The initial barrier caused by thrombosed lymphatics and coagulated serum in the tissue spaces of the inflamed area thus plays a definite rôle in immunity by protecting the essential organs at the expense of local injury.

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Trypan blue injected at the periphery of an inflamed skin area treated with a concentrated urea solution and bacteria (*Staph. aureus*) penetrates readily into the area, whereas it fails to do so when introduced around an inflamed area consequent on the injection of distilled water and bacteria (*Staph. aureus*).

Concentrated urea *per se* is an inflammatory irritant. Graphite particles injected into a peritoneal cavity previously treated with concentrated urea penetrate freely to the retrosternal lymphatic nodes; into the circulating blood accumulates rapidly in cutaneous areas almost immediately after the latter have been treated with concentrations of urea ranging from 50 per cent down to 20 per cent.

The results of this study furnish evidence, in addition to that already provided, that fixation of foreign substances is primarily due to

mechanical obstruction caused by a fibrin network and by thrombosed lymphatics at the site of inflammation. The significance of fixation in relation to immunity and its bearing upon some of the other processes involved in the inflammatory reaction have been stressed.

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## EXPLANATION OF PLATES

## PLATE 8

FIG. 1. Retrosternal lymphatic vessel draining normal peritoneal cavity of control rabbit (Experiment 5, Table I). Graphite particles injected into the peritoneal cavity disseminated freely to the retrosternal lymphatic nodes (+++).  $\times 189$ .

FIG. 2. Retrosternal lymphatic vessel draining peritoneal cavity 6 hours and 30 minutes after 20 cc. aleuronat suspension in 50 per cent urea had been introduced into it (Experiment 3, Table I). Graphite particles injected into the peritoneal cavity disseminated freely to the retrosternal lymphatic nodes (+++).  $\times 89$ .

FIG. 3. Retrosternal lymphatic vessel draining peritoneal cavity about 7 hours after it had been treated with 10 cc. of aleuronat suspension followed by 10 cc. of distilled H<sub>2</sub>O (Experiment 5, Table I). The lumen is occluded by a fibrinous thrombus containing leucocytes in abundance. Graphite particles injected into the peritoneal cavity failed to disseminate to the retrosternal lymphatic nodes (0).  $\times 81$ .

FIG. 4. Drawing of part of thrombosed lymphatic vessel (Experiment 5, Table I). Note the graphite particles in the fibrinous network and their relative infrequency within the leucocytes. No graphite penetrated to the retrosternal lymph nodes. Inflammatory reaction induced by aleuronat and distilled water about 7 hours previously. High dry magnification.

## PLATE 9

FIG. 5. Retrosternal lymphatic vessel draining the peritoneal cavity about 22 hours after it had been treated with 10 cc. of aleuronat suspension in 50 per cent urea (Experiment 12, Table I). Note the delicate fibrinous reticulum occluding the lumen only partially. Graphite particles disseminated to the retrosternal lymphatic nodes in moderate amount (+).  $\times 117$ .

FIG. 6. Retrosternal lymphatic vessel draining the peritoneal cavity about 20 hours after it had been treated with 10 cc. of aleuronat suspension followed by 10 cc. of distilled water (Experiment 9, Table I). The lumen is completely occluded by a dense thrombus characterized by areas brightly stained with eosin. Graphite particles, injected into the inflamed peritoneal cavity, were fixed *in situ*.

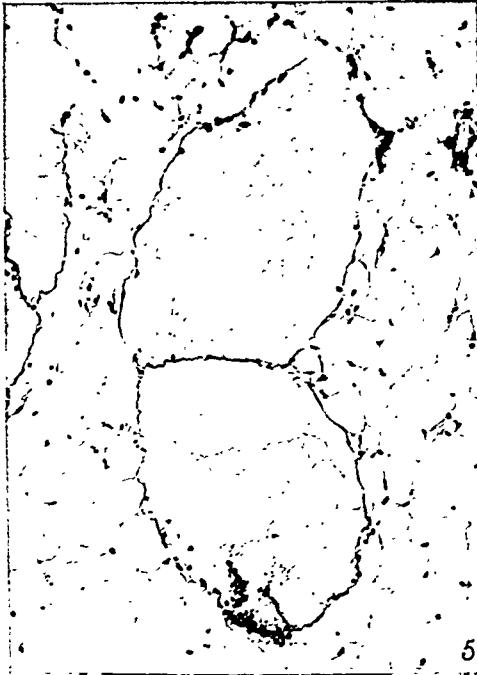
FIG. 7. Section through omentum of the rabbit whose retrosternal lymphatic is shown in Fig. 6 (Experiment 9, Table I). When graphite was injected in the peritoneal cavity, the inflammatory reaction induced by aleuronat and distilled water had already been going on for about 18 hours. Approximately 2 hours later the animal was sacrificed. No graphite had penetrated to the retrosternal lymphatic nodes and the lymphatics were thrombosed (Fig. 6). Note the deposition of graphite in the coarse fibrinous network on the omentum.  $\times 403$ .

FIG. 8. Retrosternal lymphatic vessel of Rabbit 3-68 draining its peritoneal cavity 6 hours and 32 minutes after the latter had been treated with 10 cc. of 50 per cent urea. Graphite particles are found *en masse* within a narrow fibrinous layer at the periphery of the lumen, but a sufficiently large unobstructed central area allowed free penetration of the carbon material to the retrosternal lymphatic nodes (+++). Note also red cells in the lumen, the result of a marked extravasation of blood in the peritoneal cavity.



(Menkin: Studies on inflammation, VIII)





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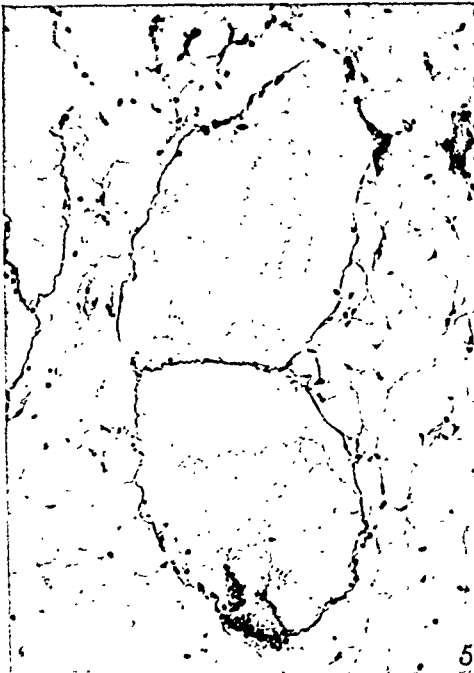
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(Menkin: Studies on inflammation. VIII)



## THE EFFECT OF LYMPHOCYTOSIS INDUCED WITH EMBRYONIC EXTRACT ON THE COURSE OF EXPERIMENTAL TUBERCULOSIS IN RABBITS

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It has long been known that individual resistance to tuberculosis is associated with activity of the lymphoid tissues. Webb and Williams (1, 2) observed a direct relationship between the lymphocyte count and the extent and activity of the disease. Soon afterward Webb, Williams, and Basinger (3) reported beneficial effects in clinical tuberculosis which they interpreted as due to the lymphocytosis induced by altitude and by passive hyperemia. Webb (4) then reported the observation of phagocytosis of tubercle bacilli by lymphocytes in cockroaches and grasshoppers. At this time, however, no differentiation had been made between lymphocytes and monocytes in the classification of the blood cells. The work of Schilling (5) and Sabin (6) has since established the fact that the monocyte is a distinct cellular entity. More recent investigations (7-9) have shown that it is this cell, not the lymphocyte, which phagocytizes tubercle bacilli. Nevertheless, further work has confirmed the belief of Webb, Williams, and Basinger, that lymphocytosis is correlated with resistance to tuberculosis. Murphy and Ellis (10) observed increased susceptibility to this disease in mice, guinea pigs, and monkeys subjected to extensive destruction of lymphoid tissues by repeated doses of X-rays. Murphy and Sturm (11) then showed that animals exposed to dry heat respond with increased numbers of lymphocytes in the circulating blood. The mechanism of this response was explained by Nakahara (12). Murphy and Sturm (13) further reported increased resistance to tuberculosis in animals showing lymphocytosis induced by heat. This observation has been confirmed by Bickford and Smithburn (14) who used rabbits, but their results were less favorable when guinea pigs were employed. The experiments of Murphy (15) tend to show that the state of lymphoid activity is the cause rather than the effect of resistance to tuberculosis.

Cunningham, Sabin, Sugiyama, and Kindwall (16) first called attention to the monocyte-lymphocyte ratio as an index to the progress and extent of the disease. They found lymphopenia to be associated with poor resistance, and that the lymphocytes in the circulation increase with an increase of resistance. That the state of tuberculous lesions in the tissues is not correlated alone with the number of

circulating monocytes was shown by Sabin, Doan, and Cunningham (17). The latter observations have since been repeatedly confirmed (18-22).

Doan and Sabin (23) recently reported experiments in which they influenced the monocyte-lymphocyte ratio of tuberculous rabbits by bringing about decreased numbers of circulating monocytes, with the result that a greater number of the animals survived into the chronic phase of the disease. It seemed advisable to study further the effect of induced lymphocytosis on the course of experimental tuberculosis. The work of Wiseman (24) has made available a simple method of inducing lymphoid hyperplasia. In the present study this method has been applied to the problem of experimental tuberculosis in rabbits.

#### EXPERIMENTAL

Twenty-five rabbits were used in the experiments. A suitable control period was allowed during which studies of the blood cells were made at frequent intervals. All differential counts were made with the supravital technique. Fifteen animals served as untreated tuberculous controls. Five animals received embryonic extract (24, 25) daily, beginning 14 days before inoculation, and in five others the injections were started 5 days after inoculation.

The embryonic extract was prepared in the following manner. Fertile eggs were incubated 7 days. The embryos were removed under aseptic precautions, crushed, and 3 cc. of freshly prepared physiological saline solution was added for each embryo. This suspension was then passed through sterile filter paper with suction and injected immediately into the animals. Injections of the extract were given into the left ear vein daily except Sunday, and in all instances except one they were continued until death. The amount of extract injected was that derived from one embryo. In the case of R 1652 the last injection was given on the 124th day of the disease.

The organisms used for inoculation were from a 10 day subculture of bovine tubercle bacilli, Strain B-1, isolated by Dr. Theobald Smith, which had been grown on Petroff's egg-gentian violet media. This strain is known to be virulent for rabbits. Each rabbit received 0.1 mg. (moist weight) of organisms suspended in 1 cc. freshly prepared normal saline in the right ear vein. Inoculations were made in groups of fifteen and ten, specific untreated controls inoculated from the same suspension being included in each group.

Studies of the blood were made once or twice weekly during the 1st month after inoculation, and once or twice monthly thereafter. Blood for counts was obtained from puncture of the marginal vein of the left ear. Autopsies were performed on all animals. The tissues were fixed in Helly's solution and the paraffin sections were stained with hematoxylin and eosin. In addition, the lymph nodes from some of the animals were stained by the method of Giemsa.

## RESULTS

The blood counts of the animals treated with embryonic extract showed both a relative and an absolute increase in lymphocytes. In addition, as Wiseman (26) has shown, there was an increase in the number of young lymphocytes in the circulation, as well as evidence of hyperplasia in the fixed sections of lymph nodes. After the control period the animals treated with embryonic extract showed lower values

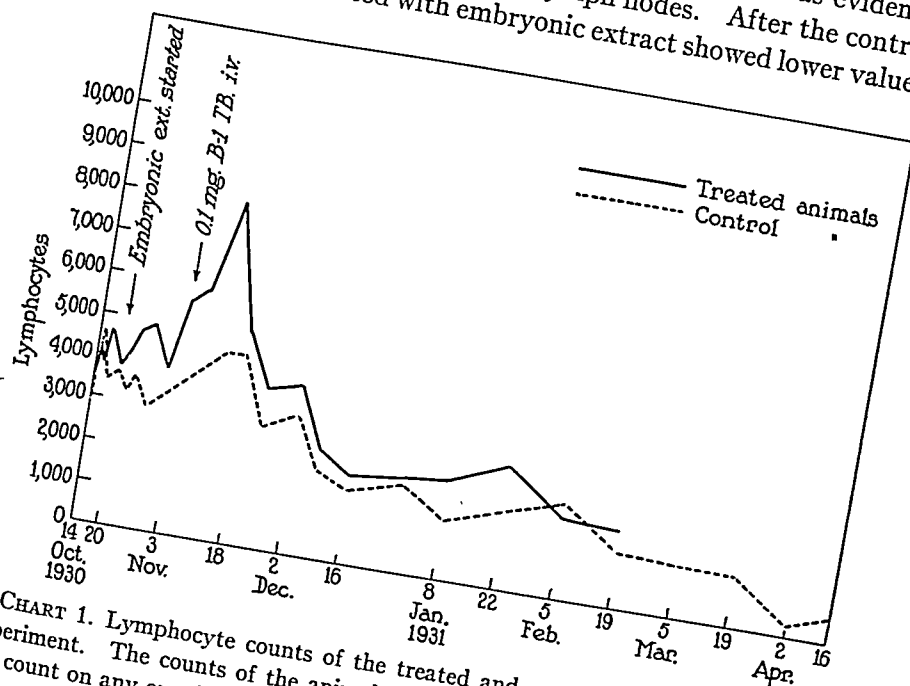


CHART 1. Lymphocyte counts of the treated and control animals in the first experiment. The counts of the animals were fused and averages taken, so that the count on any one day is an average for the group. The date of the first injections of embryonic extract and the date of inoculation are indicated. The curve for the treated animals is the shorter because the animals died earlier.

for hemoglobin and red blood cells than their specific controls. No other changes of significance occurred in the blood.

Chart 1 shows a graph of the circulating lymphocytes of the animals included in the first experiment; namely, ten tuberculous controls and five rabbits which received embryonic extract beginning 14 days before inoculation. The values on the chart represent an average for the fused counts made on any one day. It will be noted that the

treated animals showed an increase in lymphocytes to 5,500 on the day of inoculation. The number of lymphocytes continued to rise and reached a peak of more than 8,000 cells 9 days after inoculation. Thereafter a decline occurred but the treated animals continued to show higher average values for lymphocytes than the untreated tuberculous controls.

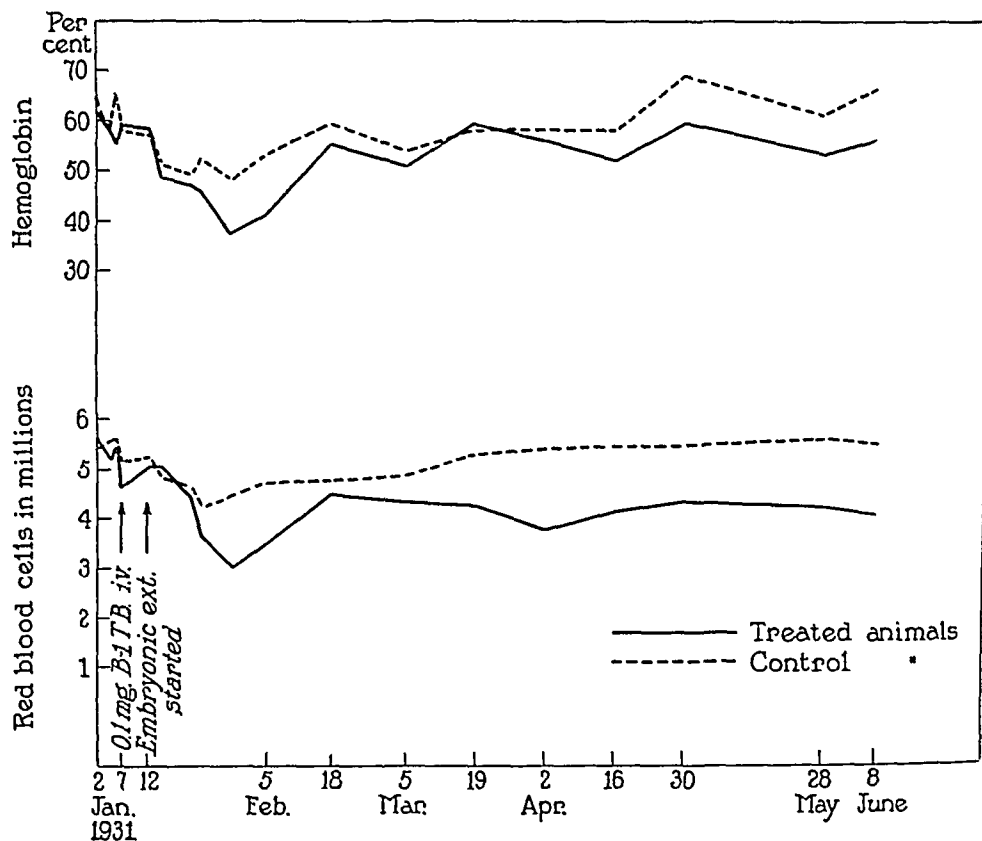


CHART 2. Values for hemoglobin and red blood cells for the animals included in the second experiment. Hemoglobin values are in per cent (Newcomer method) and red blood cells in millions per c. mm. The dates of inoculation and first injection of embryonic extract are indicated.

On Chart 2 are recorded the values for hemoglobin (Newcomer) and red cells of the animals included in the second experiment. In this experiment the five animals received embryonic extract beginning 5 days after inoculation. Here again the recorded values represent an average of the fused values determined on any one day. It will

be noted on this chart that both hemoglobin and red blood cells were lower throughout the course of the disease in the animals treated with embryonic extract.

The average survival after inoculation in the two groups of treated animals was 82.7 days, as compared with 114.26 days for the controls. However, as Thomas (27) has shown, the average survival for any group is less significant than an analysis of death rates per unit of



CHART 3. Number of animals (per cent of the whole) which died in each 15 day interval of the disease. The vertical line through the seventh segment indicates the division made by Thomas between acute and chronic phases of the disease.

time or, more specifically, the distribution of deaths through acute and chronic phases of the disease. Accordingly, on Chart 3 is shown the distribution of deaths by 15 day periods throughout the experiment (see Thomas (27), Fig. 1). It will be noted that only 20 per cent of the animals treated with embryonic extract survived more than 100 days, while more than 50 per cent of the controls survived beyond this period.



Animal No.	Survival	Extent of lesions						Adrenal	Testis	Ovary	Type of lesions			
		Lungs	Kidneys	Spleen	Liver	Bone marrow					Lungs	Kidneys	Spleen	
						Tibia	Femur							
	days													
R 1650	26	++++	+	++	+	++	++			-	Diff.	Diff.	Diff.	
R 1649	29	++++	+	+++	++	++	++	-	-		Diff.	Tb.	Diff.	
R 1651	31	+++	-	+	++	++	++	-	+		Diff.	-	Diff.	
R 1648	48	++	-		++	-	-	-		-	Diff.	-		
R 1543	53	++	-	++	+	-	-	-	++		Diff.	-	Tb.	
R 1547	56	+++	++	+	-	-	-	-	+++		Tb.	Tb.	Tb.	
R 1549	68	+++	++	+	-	++	-	-	+++		Tb.	Tb.	Tb.	
R 1541	79	+++	++	-	-	++	-	-	++++		Tb.	Tb.		
R 1544	113	+++	+++	-	-	+	+	-			Diff.	Tb.		
R 1652	324	++	++	++++	+	-	-	+		-	Diff.	Tb.	Diff.	
Untreated														
R 1653	33	++++	-	++	++	+	+	-		-	Diff.	-	Diff.	
R 1548	39	++++	+	+	-	++	++	-	+++		Diff.	Diff.	Diff.	
R 1654	48	+++	++	-	+	-	-	-			Diff.	Tb.		
R 1657	48	+++	++	+	++	++	++	+		-	Diff.	Diff.	Tb.	
R 1553	56	+++	++	+	-	-	-	-	+++		Diff.	Tb.	Diff.	
R 1545	67	+++	-		-		+	-			Tb.	-		
R 1540	79	+++	+	-	-	+	-				Tb.	Tb.		
R 1542	109	+++	++	-							Tb.	Tb.		
R 1546	113	++	+++	-	-	-	-	-	+		Tb.	Tb.		
R 1550	114	+++	++	+	+	++	++	+	++++		Tb.	Tb.	Diff.	
R 1551	141	++	+++	++++	-	++	+	-			Tb.	Tb.	Diff.	
R 1656	146	++	++	++++	-			-			Tb.	Tb.	Diff.	
R 1554	149	++	++	+++	+	++	+				Tb.	Tb.	Diff.	
R 1552	171	+++	+++		-	+	-	-			Tb.	Tb.	G.C.	
R 1655	401	++	+	+	+	-	-	-		-	Tb.	Tb.		

In Table I are presented the pathological findings. All animals with numbers under 1600 were included. The extent of tuberculosis in a given viscus, from slight to massive. Diff. indicates diffuse proliferation of tubercles. Blank spaces indicate no tissue saved. - indicates no tuberculosis found. G.C. refers to Langhans type of the nodes. Lymph reaction to tb. indicates the extent to which tubercles in the various viscera were present.

\* These are serial numbers of animals used in this laboratory covering a term of years.

Animals	Lymph nodes								Young lymphocytes in nodes	Lymphocytic reaction about tubercles	Caseation	Giant cells	Tuberculosis in other organs
	Inguinal	Popliteal	Axillary	Cervical	Submaxillary	Mesenteric	Retrosternal	Tracheal					
1	+++	+++	++			+++			+++	+	++	+++	Heart
2	++	+++	+++	+	+	++		++++	+++	++	++	++	
3	++	++	-		+	+			++	+	+	+	
4	++	+	++			++			+	++	+	+	
5	+++	+++	+++	+		+++			++	++	+	+	Ear Spinal cord Eye
6	-	-	-		-	+		+	+	++	+	++	
7	-	-	+			+		+	++	++	++	++	
8	-	-	-		-	+		+	+	+++	+++	+++	
9	-	-	-		-	++	-	+	No	++	++	+	
control animals													
10	+++	++	++		++	++			++	+	++	+	Omentum
11	+++	+++			+++	++			+	+	+	+	Heart
12	+	++	+		-	-			+	+	+	+	
13		+++	+++		++	+		++	+	++	+++	+++	
14	-		+			+			No	+	+	++	
15	-	+++	++			++	++		+	++	++	+++	Omentum Appendix; ileo-cecal valve; mammary glands
16	-	-	+		++	+			+	++	+++	++	
17	-	+	+		-	+			No	++	+++	++	
18	-	-	-	+	++	-			No	+	++	+	
19	-	-	+		+	+			No	+	++	+	
20	-	-	-		-	-			No	++	++	+	
21	-	-	-		-	-			No	+++	++	+	
22	-	-	-		-	-	+		No	+++	+++	+	
23	-	-	-		-	-	-		No	+++	+++	++	
24	-	-	-		-	-	-		No	+++	+++	++	

st experiment; all with numbers over 1600 were in the second experiment. + to ++++ indicates the  
s without formation of discrete tubercles. Tb. indicates lesions which were discrete and circumscribed.  
ls. By young lymphocytes is intended an estimation of the relative numbers of immature lymphocytes in  
mphocytes.

Interesting observations were made in the study of the stained sections. As has been pointed out by Doan and Sabin (28) and more recently by Thomas (27), the animals dying in the early or acute phase of experimental tuberculosis show extensive, widespread, diffuse involvement of the bone marrow, lymph nodes, spleen, and lungs. Thereafter, as the above investigators noted, there is gradual regression of the lesions in these organs; and throughout the disease significant changes in the blood cells occur which reflect the altered pathological picture. In Table I are recorded the distribution, character, and roughly the extent of the tuberculous lesions occurring in the various organs of the 25 rabbits in these experiments. The treated and control animals are arranged in this table in the order in which they died. The changing pathological picture of experimental tuberculosis described by Doan and Sabin and later by Thomas may be observed in the study of this table. It has also been observed that lesions in the testes and eyes tend to occur late in the disease; that renal lesions become more extensive as time goes on; that the adrenal and ovary are not frequently involved; that lesions in the liver are usually not extensive and often consist of isolated Langhans or rosette giant cells alone; and that generally speaking, tuberculous lesions in any organ are first diffuse, later becoming discrete and circumscribed.

Attention has been called by several investigators to the early increase in circulating monocytes with progression of tuberculosis in the various organs. In addition to the gradual upward trend in the number of monocytes, frequently there are showers of these cells in the blood stream; and the monocytes in such instances are often modified or stimulated and approach epithelioid cells in appearance. A reasonable explanation for such a phenomenon has been observed in the study of stained sections from some of the animals dying in the early stages of this experiment. Rabbits R 1649, R 1651, and R 1654, which died on the 29th, 31st, and 48th days respectively, each showed intravascular tubercles in the vessels of the lungs, in one instance in an arteriole, in the others in veins. In a few of these intravascular lesions the epithelioid cells lay beneath the vascular endothelium; in other instances vascular endothelium was not to be seen, the tuberculous tissue being exposed to the circulating blood. A few of the smaller veins in some of these sections were completely occluded by

tuberculous thrombi. It is quite possible, indeed probable, that some of these cells may be swept free in the blood stream and account for the highly stimulated forms of monocytes or even epithelioid cells. It is quite conceivable also that if a large clump of cells were swept into the blood stream as an embolus it might account for some of the deaths occurring early in the disease when thrombosis is present.

Ropes (30) has shown that the columnar or cuboidal cells of tracheal and bronchial epithelium exhibit staining reactions similar to phagocytic cells, and themselves possess phagocytic power. Three animals in this series, R 1548, R 1650, and R 1653, exhibited areas in the sections in which there was marked proliferation of bronchial epithelium and in which bronchial epithelial cells were apparently in process of fusion to form giant cells. The cytoplasm of the latter was foamy, giving the appearance of phagocytic activity. It will be noted that these animals died on the 39th, 26th, and 33rd days respectively, at times that is to say, when the extent of disease in the lungs was near a maximum.

An effort has been made to estimate the state of activity of the lymph nodes on the basis of the numbers of young lymphocytes in the sinuses, cords, and follicles, the size of the follicles and the number of mitoses in them. These observations are recorded near the right side of Table I and, as can be seen, they bear no direct relation to the number of lymphocytes seen about tubercles (recorded in the next column), or to resistance. However, there is apparently a direct correlation between the activity of the lymph nodes and the extent of tuberculosis in these structures. Indeed, it appeared as if the germinal centers of the nodes were the site of election for tubercle formation.

In the lymph nodes the earliest involvement is exhibited by the presence of epithelioid cells in the peripheral sinuses. These cells are scattered diffusely and rarely occur in clumps forming tubercles. The germinal centers of the follicles next become involved, after which the lesions spread in all directions from the germinal centers. In numerous lymph nodes the germinal centers alone are involved. Moreover, nodes which show the greatest hyperplasia, that is, the most reversion to the primitive cell type, show most extensive tuberculosis in the germinal centers. It is as if the disease reaches the

lymph nodes through the lymphatic vessels and, having gained access to the node, resides and progresses in the place where the primitive cells are most abundant. It seems reasonable, then, to suppose that the primitive cell which normally gives rise to the lymphocyte may, on occasion, differentiate into a monocyte or epithelioid cell and function as such.

It is also interesting to note that the mesenteric nodes were the most frequently involved, which seems almost paradoxical in view of the observations of Forkner (29) that monocytes are rarely seen in these nodes from normal animals. In the present experiments tubercles were seen in 70 per cent of 51 lymph nodes from animals treated with the embryonic extract, as against 49 per cent of 69 nodes from the specific controls. Since in young individuals there is greater activity of lymph nodes than in older persons, there appears to be some similarity between the lymphoid tuberculosis of the treated animals in these experiments and the tuberculosis of children, which most frequently involves the lymphatic system.

It may be that when lymphocytosis is accompanied by increased resistance the age of lymphocytes is an important factor, or that lymphocytosis is an incidental expression of some influence directly responsible for resistance. Further experiments are in progress, which it is hoped will elucidate these points.

#### SUMMARY

1. Chick embryo extract given intravenously did not favorably influence the course of experimental tuberculosis in rabbits, although it did cause lymphocytosis and lymphoid hyperplasia.

2. The tuberculous animals treated with chick embryo extract showed lower values for hemoglobin and red blood cells than tuberculous controls inoculated from the same suspension.

3. Under the conditions of these experiments an increase in the number of young, immature cells in the lymph nodes was accompanied by more extensive tuberculous lesions in the lymph nodes and especially in the germinal centers of the nodes. A possible analogy to lymphatic tuberculosis in children is suggested.

4. The observations of previous investigators on the progression and later regression of lesions in experimental tuberculosis have been

confirmed. The distribution, character, and extent of lesions have been studied and are presented in tabular form.

5. The presence and significance of tuberculous thrombi in the lungs are discussed.

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# THE DIPHASIC NATURE OF TUBERCULOSIS IN RABBITS AFTER INTRAVENOUS INOCULATION WITH BOVINE TUBERCLE BACILLI

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Studies with reference to the correlation existing between the cellular reactions in the tissues in experimental tuberculosis and the changes in the circulating blood cells have been carried on in this laboratory since 1925. Observations on the connective tissue cells have been made, both with the supravital technique and with fixed tissues prepared by the standard methods (1). General studies of the changes in the blood cells associated with changes in the lesions during the course of the disease have been reported (2, 3) and certain changes in the blood cells directly related to the pathological findings in the bone marrow have been described. Since these observations were reported, a considerable number of animals have been inoculated with tuberculosis as specific controls for groups of animals under various treatments. The accumulated mass of data relevant to the course of tuberculosis in rabbits is reviewed and analyzed in the present paper.

## *Material and Methods*

The changes in the blood cells following inoculation with tuberculosis have been followed in a group of 175 animals. 140 of these received no treatment and were allowed to die of tuberculosis. The remaining 35 were either killed for comparative studies or were given some treatment, in which latter instance no further studies of their blood were included in this analysis. From the group of 140 animals, sections were available for study in 109 instances.

The animals used have been of various breeds and weighed 2,000 gm. on the average. Male and female rabbits have been employed in approximately equal numbers. The season of the year in which inoculations were made was not limited, groups of animals having been inoculated in every month except July and August. An analysis of the records showed that the course of the disease in



animals inoculated in June or September was similar to that observed in animals inoculated in December or January. They were kept under uniform housing and dietary conditions, and during the period that they were in our animal house there were no acute infectious diseases of rabbits which assumed epidemic proportions. There were sporadic cases of severe snuffles, and some cases of pneumonia of undetermined origin. An occasional animal showed evidence of spontaneous encephalitis. The effect of these isolated instances of deaths due to accessory diseases, on the general study of mortality rates from tuberculosis is slight.

The organism used was a strain of bovine type tubercle bacillus designated B-1 which has been cultivated on artificial media since its isolation from a bovine lesion by Dr. Theobald Smith in 1893. It has been virulent for rabbits during the 6 years that it has been in use in this laboratory. The organisms were harvested from colonies grown on Petroff's egg media and weighed directly. They were then triturated in 0.9 per cent sodium chloride solution and suspended so that the infecting dose was contained in a suitable quantity of fluid, 0.5 or 1.0 cc. All animals were inoculated in the marginal ear vein, the dose varying from 0.001 mg. in the case of four animals up to 2.0 mg. In the past 2 years 0.1 mg. has been used as the infecting dose.

During the course of the disease the animals were weighed and the temperature taken on the day the blood counts were made. The intervals between counts varied in the different groups but in general the animals were counted at weekly intervals for the first 2 months after inoculation and at 2 to 4 week intervals thereafter. At death the animals were autopsied and blocks were saved from each organ for sectioning.

### *The Rate of Mortality in Tuberculous Rabbits*

The mortality rate in a population subject to an acute infectious disease usually follows a course somewhat as follows:—

There is at first a comparatively low rate of mortality which precedes a period during which the mortality increases rapidly and reaches a peak; following this there is a decline in the rate of mortality which is less rapid than the increase prior to the peak. When the total mortality of such a group is plotted against time, the curve assumes a sigmoid shape, skewed more or less toward the later time periods. This distribution of mortality is interpreted as reflecting the distribution of susceptibility in the whole group. The early deaths are attributed to the greater susceptibility of a portion of the population, the larger portion succumbing at or near the point of peak mortality representing the members of average resistance.

In Fig. 1, the actual number of deaths per 15 day period is shown for the group of 140 animals mentioned above. It is seen that there

were two definite peaks in the mortality rate, one occurring at the end of the 1st month, and the other occurring at the 5th month, with an intervening period at the end of the 3rd month, when the mortality rate was much lower than the general rate for the entire period. In

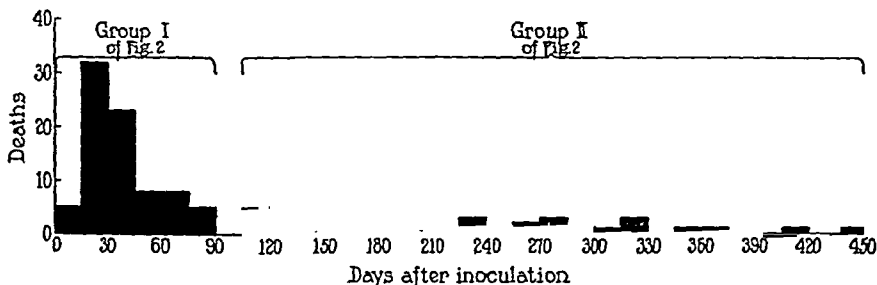


FIG. 1. Death rates during 15 day periods after inoculation in 140 tuberculous rabbits.

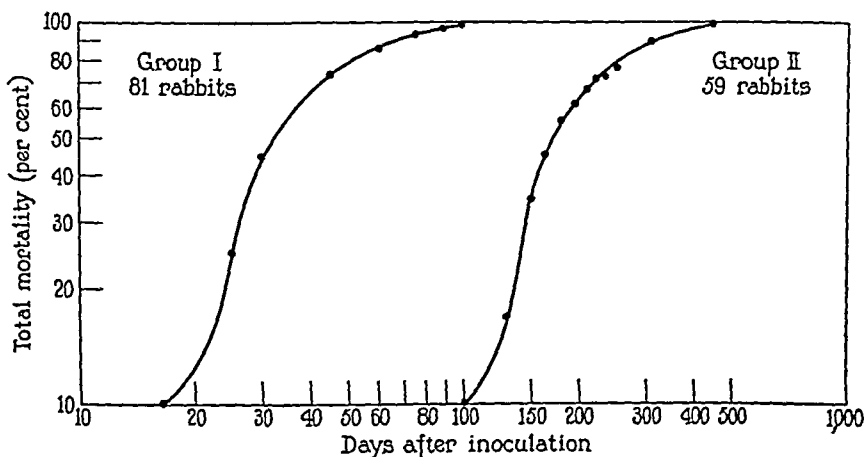


FIG. 2. Total mortality of 81 rabbits which died under 100 days (Group I) and 59 rabbits which died between 100 and 437 days (Group II).

this series no deaths occurred in the period from 85 to 100 days. In Fig. 2, these data are rearranged so that the total mortality of each of the two groups of animals, *i.e.* those dying before 85 days and those dying after 100 days, is shown. The per cent of animals dead is plotted on the ordinate and the time after inoculation along the

animals inoculated in June or September was similar to that observed in animals inoculated in December or January. They were kept under uniform housing and dietary conditions, and during the period that they were in our animal house there were no acute infectious diseases of rabbits which assumed epidemic proportions. There were sporadic cases of severe snuffles, and some cases of pneumonia of undetermined origin. An occasional animal showed evidence of spontaneous encephalitis. The effect of these isolated instances of deaths due to accessory diseases, on the general study of mortality rates from tuberculosis is slight.

The organism used was a strain of bovine type tubercle bacillus designated B-1 which has been cultivated on artificial media since its isolation from a bovine lesion by Dr. Theobald Smith in 1893. It has been virulent for rabbits during the 6 years that it has been in use in this laboratory. The organisms were harvested from colonies grown on Petroff's egg media and weighed directly. They were then triturated in 0.9 per cent sodium chloride solution and suspended so that the infecting dose was contained in a suitable quantity of fluid, 0.5 or 1.0 cc. All animals were inoculated in the marginal ear vein, the dose varying from 0.001 mg. in the case of four animals up to 2.0 mg. In the past 2 years 0.1 mg. has been used as the infecting dose.

During the course of the disease the animals were weighed and the temperature taken on the day the blood counts were made. The intervals between counts varied in the different groups but in general the animals were counted at weekly intervals for the first 2 months after inoculation and at 2 to 4 week intervals thereafter. At death the animals were autopsied and blocks were saved from each organ for sectioning.

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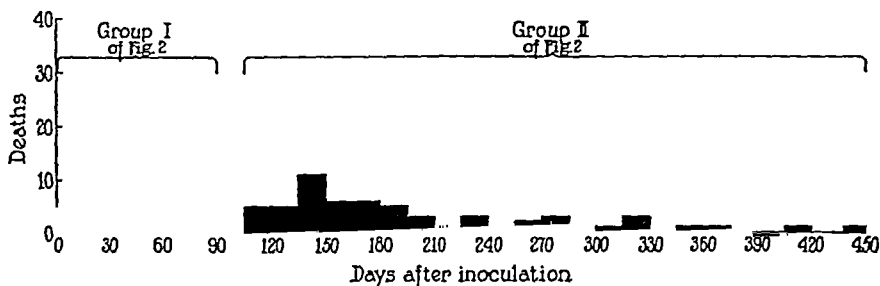


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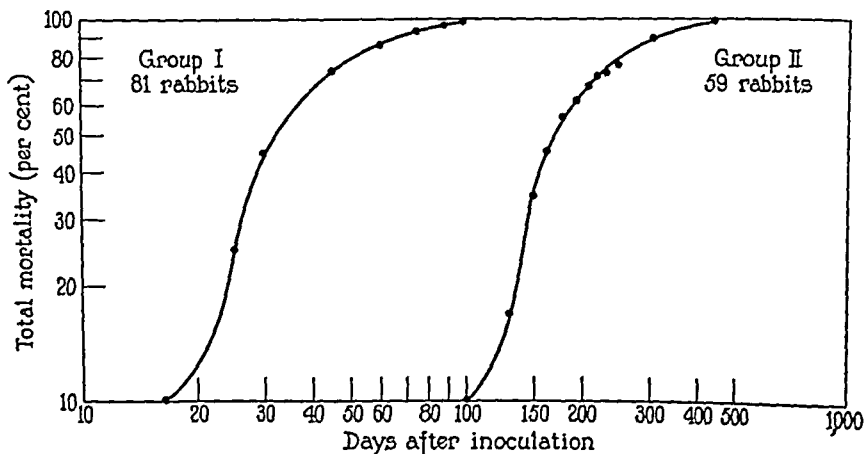


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abscissa. Both sets of values are plotted as logarithms. It is obvious that the mortality for the whole group of 140 animals is in reality the summation of the mortalities occurring during two separate phases of the disease, and that in each instance the distribution of deaths is similar to that described above as typical of an acute infectious disease. The effect of these two separate mortality pressures has been evident in groups varying in size from 5 animals to 40. It has been seen also with all doses used save the 0.01 mg. and 0.001 mg., that a large dose resulted in a greater number of animals succumbing in the first phase of the disease, while a small dose allowed a greater number to survive the first phase. In the case of the animals which received 0.01 and 0.001 mg., four in each case, all survived the first phase of the disease.

It would seem therefore that if the resistance of rabbits to tuberculosis initiated through intravenous inoculation is to be measured by the longevity of the animals inoculated, it must be expressed in terms of resistance to two distinct phases. The use of an average obtained by dividing the sum of all the days lived by the number of rabbits in the group, may be quite misleading, since the distribution of deaths in two groups might be significantly different and yet result in the same average.

### *Differences in Lesions in the Two Phases*

The sections of 109 animals were found to be suitable for study. The extent and type of the lesions were noted and the findings were then grouped according to the longevity of the animal. The pathological picture during successive 15 day periods was then summarized.

#### *A. Lesions in the First Phase*

*The Lungs.*—A tuberculous pneumonia was found in almost every animal that died during the 1st month after inoculation. The alveoli were filled with an exudate made up of monocytic and epithelioid cells. The alveolar walls were thickened and showed monocytic cells in and adherent to them. There were some polymorphonuclear leucocytes and clasmatoocytes present. Exudation of red cells and fibrin was rarely noted. In the gross the lungs were of normal color, but did not collapse completely when the thorax was opened. The surface frequently showed a number of small, pearly gray tubercles, with translucent borders, 1 to 2 mm. in diameter. On gentle pressure of the lungs a small amount of exudate

could be expressed from the trachea. Caseation of portions of the pneumonic tissue was seen at this time. In those animals which died between 13 and 20 days, these lesions were quite pronounced; however, at the end of the 1st month many animals showed practically the whole lung consolidated with pneumonia. During the 2nd and 3rd months the extent of the pneumonic involvement decreased steadily, and in those animals which died at the end of the 3 months only small scattered collections of epithelioid cells could be found. The classic, fully developed tubercle, formed of epithelioid cells and giant cells, enveloped by lymphocytes and fibrous tissue, was rarely present in the 1st month; however, in the 2nd and 3rd months they were present in increasing number together with Langhans giant cells free in the alveoli. In Fig. 3, the occurrence of these various lesions has been plotted. The occurrence of tuberculous pneumonia, and of fully

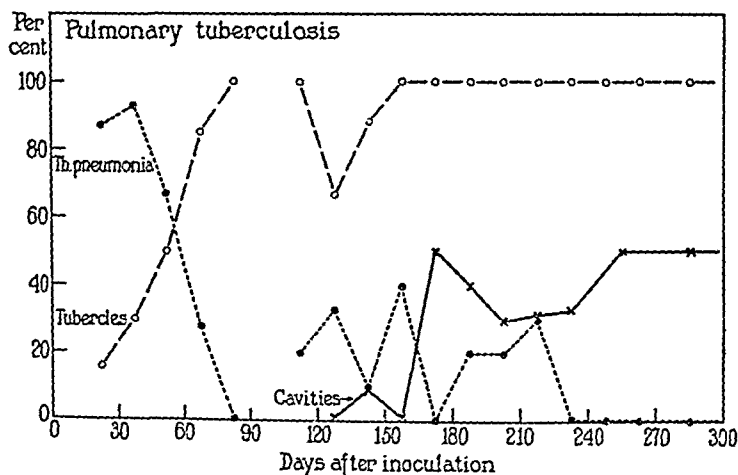


FIG. 3. The distribution in relation to time of various pulmonary lesions.

developed tubercles, is plotted as per cent for successive 15 day periods. No attempt was made to estimate the total extent of all types of lesions, as sections taken one from each lobe do not give sufficient data for such a comparison. It is seen that the incidence of tuberculous pneumonia decreases rapidly after the 1st month, while the incidence of fully developed tubercles increases steadily from the 1st month onward. It seems highly probable that the small amount of exudate found at the end of the 3rd month is in part residual, since the injection of dead tubercle bacilli intravenously is followed by the appearance of a caseous pneumonia in a very short time, and small collections of epithelioid cells entirely similar to those mentioned above may persist in the lungs for as long as 60 days. It is probable that the infection with living, virulent organisms results in the production of large amounts of exudate in which bacilli are either killed or fail entirely to multiply and which is removed from the lung slowly. Any attempts to

estimate the state of activity of a particular lesion by study of the histologic sections alone must take this factor into consideration.

*The Spleen.*—In the first 4 weeks the spleen was involved in almost every instance. The extent of the monocytic and epithelioid cell reaction was greater in the spleen at this time than in any other organ. In many cases the normal histologic appearance of the organ was completely obliterated, both pulp and follicles being invaded. The weight of the organ was increased; the average weight of the spleen at this time was 3.2 gm. as against a normal average of 0.9 gm. In one instance the weight of the spleen was 17 gm., and section showed the entire organ to be filled with monocytes and epithelioid cells. Congestion of the spleen with blood was not seen. During the 2nd and 3rd months, however, the spleens examined showed a marked diminution in the extent of the lesions, going on to com-

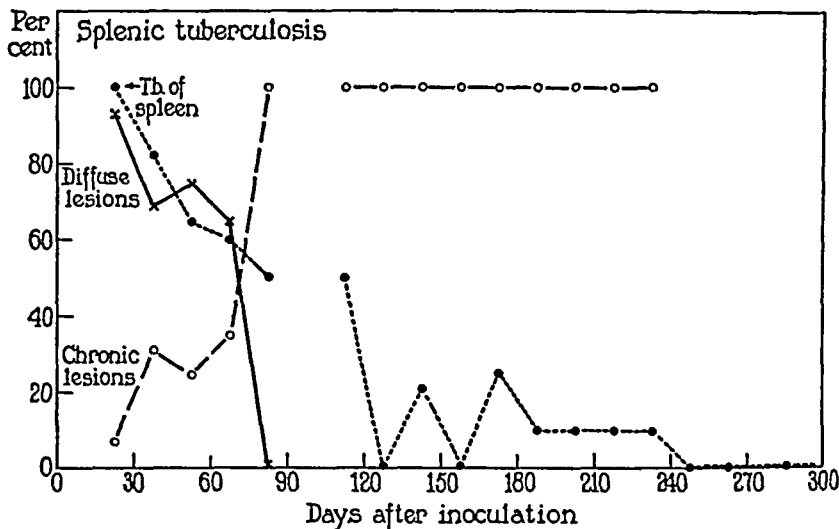


FIG. 4. The distribution in relation to time of lesions of the spleen.

plete healing in many instances. This process of healing did not seem to have taken place by the caseation of a large mass of the tuberculous tissue with fibrosis of the cavity formed, but by the elimination of the cells by absorption and possibly by phagocytosis by clasmotocytes. The epithelioid cells in these regressing lesions lost their staining reaction and their normal morphology; the nuclei and cytoplasm both appeared degenerated and in many instances only faintly stained cell outlines could be made out. Lymphocytes and polymorphonuclear leucocytes were seen in and around such areas, possibly aiding in the complete removal of such cells. In but a few instances localized tubercles were seen at the end of the 3rd month. In Fig. 4 the number of spleens in which tuberculous lesions were found is plotted and shows a steady decline after the 1st month. The number in which diffuse lesions were found is expressed in per cent and is seen to decline rapidly after the 1st month. Small, localized tubercles consisting most often of a few epithelioid

cells or an occasional Langhans giant cell were classed as lesions of a more chronic nature and their increase is shown in Fig. 4.

*The Bone Marrow.*—The course of the tuberculous infection in the bone marrows of a large group of rabbits has been studied and reported by Doan and Sabin (2). They demonstrated that there were no significant changes visible in the marrow before the 8th day, when a definite infiltration with monocytes and epithelioids was seen. At this time the fat was seen to be breaking up and disappearing, and from the 8th day on up to the end of the 4th week, the marrow showed an increasing amount of the diffuse epithelioid cell reaction. In a few animals that survived longer than 30 days, they reported a steady regression of the lesions to complete healing at the end of 3 months.

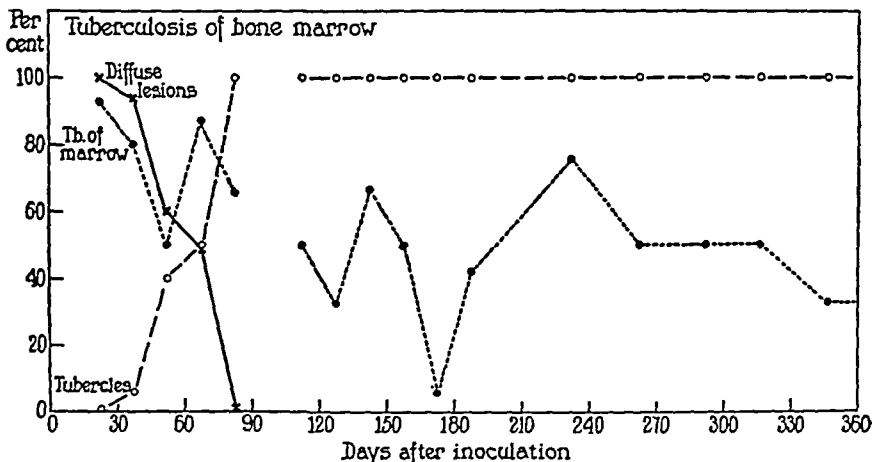


FIG. 5. The distribution in relation to time of lesions of the bone marrow.

In the present series of animals similar findings were noted in the marrows of those succumbing in the 1st and 2nd months. At the end of the 3rd month about half of the animals showed no tuberculous involvement of their marrows whatever, while the remainder revealed varying numbers of small, definitely localized tubercles scattered throughout the tissue. These relations are shown in Fig. 5.

*The Lymph Glands.*—In the 1st month after inoculation, almost every lymph node showed tuberculous involvement. The lesions consisted of a diffuse infiltration of the entire node with monocytes and epithelioid cells. Caseation was found in a considerable number. During the 2nd and 3rd months, however, the incidence of tuberculous lymph nodes decreased and the lesions were definitely less extensive. The phenomenon of regression was noted in a considerable number, similar in appearance to the regression of the lesions in the spleen and bone marrow. Caseation of tuberculous nodes was much less frequent in the 2nd and 3rd months than in the 1st. Definitely localized tubercles did not make their



appearance in the 2nd and 3rd months, although giant cells were more frequent. The lesions at the end of the 3rd month resembled those seen in the spleen, where small collections of epithelioids and giant cells remained with no evidence of fibrous tissue forming about them.

*The Liver.*—In the liver, tuberculous lesions apparently mature more rapidly than in the other organs, as in this series of animals the early reaction of monocytes and young epithelioid cells was not observed. The lesions found were constant in type, namely, a scattering of Langhans giant cells throughout the organ, occurring most often singly, but occasionally in groups of two or more. Had animals been studied during the first 2 weeks after inoculation a much more active type of reaction would probably have been observed. The number of animals showing any involvement of the liver during the 1st month was 60 per cent, and this figure decreased rapidly in the ensuing months.

*The Kidneys.*—Renal tuberculosis was found to exist in about 70 per cent of the animals that died in the 1st, 2nd, and 3rd months. This rate of incidence did not lessen as time went on, a fact which would suggest that healing of the renal lesions once under way is uncommon. The lesions in the kidneys were for the most part macroscopic tubercles, located both in the cortex and in the medulla. They tended to extend between the tubules, and frequently made their way into the pelvis of the kidney, where tuberculous pyelitis was set up. When the pelvis was so involved, there was evidence of fresh invasions of the medulla from the pelvis. It was common to find the pelvis completely filled with caseous debris. Epithelioid cells were occasionally seen in the glomeruli, and some small tubercles which evidently had their origin from such a lesion. Histologic sections of the ureters and bladder were not made, but no gross tuberculous involvement of these portions was observed.

*The Reproductive System.*—In but two cases were tubercles found in the testicles of animals that died in the acute phase; these two were small, pin-point tubercles without caseation. No ovarian tuberculosis has been recorded.

### *B. Lesions Found in Animals Surviving beyond 3 Months*

*The Lung.*—In the second or chronic phase of the disease all of the changes associated with long standing tuberculosis were found in the sections. Tubercles of varying size and in varying stages of development were found. Large confluent tubercles which had apparently extended into adjacent lung tissue were in some cases walled off and in others surrounded by a zone of tuberculous pneumonia. Many small tubercles were seen to be well enveloped by lymphocytes and fibrous tissue. It was noteworthy that in general the greater the longevity of the animal the more sharply localized were the lesions; and the rest of the lung showed less and less evidence of any inflammatory reaction, in sharp distinction to the findings in the acute phase where the whole lung from apex to base was frequently involved. In those animals which survived a year or more, the extent of the lesions was markedly reduced, and in some instances confined to the presence of a few small cavities with dense fibrous walls.

*The Spleen.*—The spleen was seldom found to be tuberculous in the chronic phase of the disease. Occasionally a few scattered epithelioid cells or Langhans giant cells were observed. No large confluent tubercles were found. In a number of spleens, particularly from animals that died after 5 or 6 months, there was a marked change in the histologic appearance, involving a necrosis of the cells of the pulp and much fibrosis; the lymphoid follicles were remarkably reduced in size. The average weight of the spleen was approximately normal, but in a considerable number of cases the organ was very small and shrunken.

*The Peripheral Lymph Nodes.*—The incidence of tuberculous lymph nodes during the chronic phase of the disease decreased steadily. The nodes found in animals surviving over a year were in general very small and difficult in many cases to locate at autopsy, but apparently normal in other respects. The popliteal lymph node was more often involved than any other peripheral node, and an analysis of the records showed this to be associated with tuberculous infection of the bone marrow of the tibia and femur. The incidence of the two lesions together was frequent, while the occurrence of either lesion alone was not common.

*The Bone Marrow.*—Of the animals dying in the chronic phase of the disease, about half had tubercles in the marrow. These tubercles were discrete, usually small, and made up of from 5 to 25 epithelioid cells in section. The tubercles often had a lymphocytic reaction about them, but none showed any evidence of an increase in fibrous tissue. Caseation was not seen, and there was no evidence of any inflammatory reaction in the adjacent marrow.

*The Kidneys.*—The kidneys were tuberculous in about 65 per cent of the animals that died in the chronic phase of the disease. The extent of the lesions was perhaps greater in some of the animals which had survived for a long time, but the nature of the lesions was essentially the same as that described in the acute phase of the disease. There was in no instance any evidence of regression or repair of the renal lesions.

*The Liver.*—In the chronic phase of the disease the incidence of hepatic tuberculosis was very low. Only about 15 per cent of the animals showed any lesions at all and these consisted of an occasional Langhans giant cell.

*The Reproductive System.*—Tuberculosis of the testicle was seen in a number of instances in animals that died in the chronic phase of the disease, and in these the whole organ was caseous in each case, frequently involving the epididymis.

### *C. Comparison of the Lesions in the Two Phases*

The two phases of the disease are characterized by different pathological findings. In the first 3 months the spleen, bone marrow, lymph nodes, and the liver were extensively involved, as well as the lung and kidney. In animals dying after 3 months the lesions in the spleen, bone marrow, lymph glands, and liver were minimal, whereas the lesions in the lung and kidney were extensive. In addition, the lesions

in the first 3 months were most frequently a diffuse infiltration of the organs with a mass of monocytes and epithelioid cells, which showed a tendency to regress without caseation, while in the later phase the formation of typical tubercles together with cavitation and fibrosis went on to the exclusion of any other type of lesion.

In connection with these studies the work of Lurie is most valuable and interesting. In 1928 (4) he made an extensive study, following the course of tuberculosis in rabbits after intravenous inoculation with bovine and human type bacilli, with especial reference to the number of organisms which could be recovered from the various organs. He sacrificed animals at varying intervals after inoculation, making cultures from small weighed portions of spleen, liver, bone marrow, and lung. The number of colonies that grew out during 10 weeks' observation of the cultures was determined, and the colony counts from the various organs compared. The data as presented showed that there was apparently a lag period of approximately 10 days, as cultures made from animals sacrificed 8 days after inoculation showed either no growth or a very few colonies, while cultures obtained from animals sacrificed after 2, 3, and 4 weeks showed increasing numbers of positive growths as well as colonies. Cultures made from the spleen revealed growth earlier and in greater profusion than those from the other organs. After the 4th week the number of colonies obtained from the spleen, liver, and bone marrow declined, while the number of colonies obtained from the lung and kidney increased with time, the lung showing a greater increase and the kidney but a slight increase.

The number of bacilli which can be grown from an organ is dependent on the rate of multiplication and destruction of organisms in that organ and on the number of organisms originally seeded into it; the estimation of the number of viable organisms throws no light on the ratio of living to dead bacilli. The fact that dead organisms produce lesions similar to those produced by living virulent organisms serves to complicate the pathological studies. However, lesions produced by dead organisms do differ from the active lesions in that the former show regression, a phenomenon which is clearly recognizable in sections and which may be easily followed in rabbits inoculated with heat-killed tubercle bacilli. It is probable that the regressing lesions seen in the 2nd and 3rd months in the spleen and bone marrow and lymph

glands were due to the large number of dead organisms present in those tissues after the 1st month. The rise and fall of the number of organisms recovered by Lurie from the different organs occurs coincidentally with the period of increasing and decreasing lesions in our series, and parallels the rise and fall of the death rate during the first 2 months.

More recently Lurie (5) has made studies similar to those referred to above but in which sections of tissue adjacent to the block of tissue taken for culture were fixed and sectioned, and a correlation between the bacteriological and the histological findings determined. The sequence of pathological changes reported in this recent study is substantially in agreement with the course of the changes reported in our series for the first 3 months. Lurie made a more intensive study of the events occurring up to 2 months after inoculation, recording but a few instances of studies on animals allowed to live longer.

All of the factors brought out so far in the first phase of the disease, including the mortality rate and the nature and extent of the lesions, are significantly related to the widespread multiplication of the tubercle bacilli in the various organs and their subsequent partial destruction. While the trend of total mortality in the second phase sets this portion of the disease apart as an entity, it is not so apparent that the rise and fall of the death rate in this phase is related to or dependent upon the same factors.

### *The Changes in the Blood Cells in the Two Phases*

During the first 3 months after inoculation, the lesions were widespread and constantly changing in extent in the various organs. The clinical condition of the animals during this period was apparently normal in almost every instance, and those animals which died showed little evidence of wasting or malaise. The younger and lighter animals, in fact, frequently gained weight steadily during this period. The changes in the blood cells, however, reflected faithfully the changes in the lesions in each animal as well as for the whole group. The striking correlation between lesions in the bone marrow and the blood cell changes is evident in Fig. 6, where the total number per c.mm. of the various cells is plotted by 10 day periods after inoculation. 175 animals were included in this summation, which was extended to the 190th day. The number of counts available from ani-

## DIPHASIC NATURE OF TUBERCULOSIS

Mean of blood counts at intervals of 10 days  
175 tuberculous rabbits

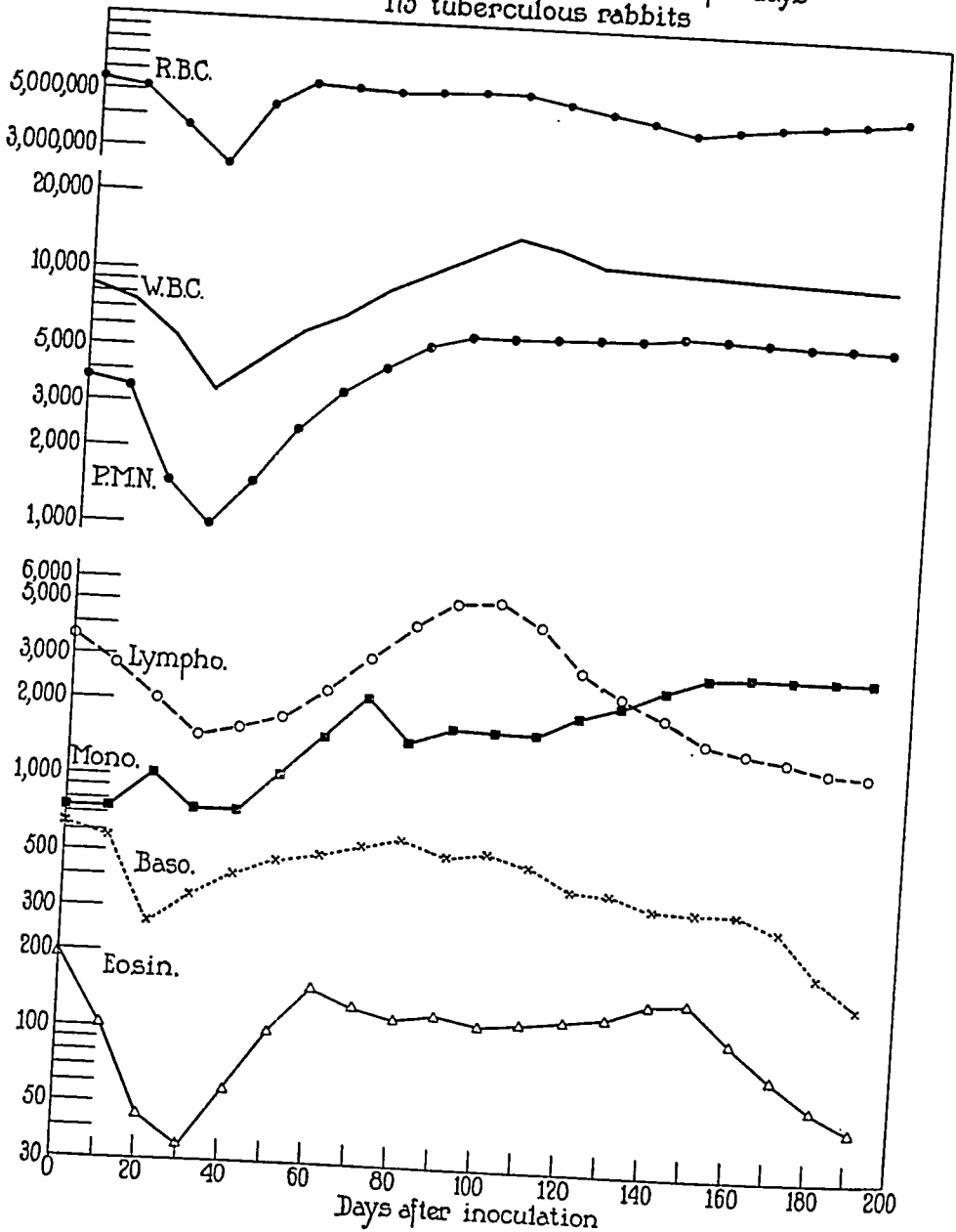


FIG. 6. The total number of the various types of blood cells per c.mm. during successive 10 day periods after inoculation, in a group of 175 tuberculous rabbits.

mals surviving beyond this time was too small to allow a significant average to be taken. It is seen that at the end of the 1st month there was an anemia and a leucopenia of all cells except the monocytes. This fall in total numbers of these cells coincided with the increase of lesions in the bone marrows, shown in Fig. 5.

Following this period of anemia and leucopenia, the cells tended to return to their previous levels, during the 2nd and 3rd months. Again this change coincided with the period in which the bone marrows were recovering from the extensive lesions present in the 1st month.

The lymphocytes fell in total numbers during the 1st month and recovered during the 2nd and 3rd months. This fall in total numbers is apparently due more to a drop in the total number of intermediate and large lymphocytes than to a proportionate decrease in all three types of lymphocytes. The lesions in the lymph glands and spleen, extensive in the 1st month, regressed in the 2nd and 3rd months, and undoubtedly these changes are related directly to the variations in the number of lymphocytes in the circulating blood. The difficulty in estimating the extent of the lesions in all of the possible sources of lymphocytes makes a close correlation between the qualitative changes in the cells and the pathological changes in the lymphoid tissues difficult to obtain.

The monocytes showed a small rise in total numbers during the 1st month. The most significant change in this cell type was a qualitative one and it is brought out sharply when the monocytes are differentiated into normal and "stimulated" forms.

The stimulated form of the monocyte seen in tuberculosis differs from the normal monocyte in the following characteristics. The only nuclear change is a greater tendency on the part of the stimulated cells toward amitotic division, so that more of the stimulated forms show two nuclei. The essential changes are cytoplasmic and consist in an increase in the size of the rosette of vacuoles staining with neutral red, correlated with an increase in the size of the whole cell. In the stimulated forms the individual vacuoles of the rosette are smaller but much more numerous. The normal monocyte has a small rosette of vacuoles of moderate size, while the epithelioid cell has a very large rosette of tiny vacuoles. The stimulated form of monocyte is intermediary between the two, and the transition from monocyte to

stimulated monocyte ending with the epithelioid cell has been demonstrated by Sabin (6). It is significant that, during the period when stimulated monocytes are found in great numbers in the circulating blood, epithelioid cells, identical with those found in the lesions, are occasionally found in the blood (7, Plate 26,

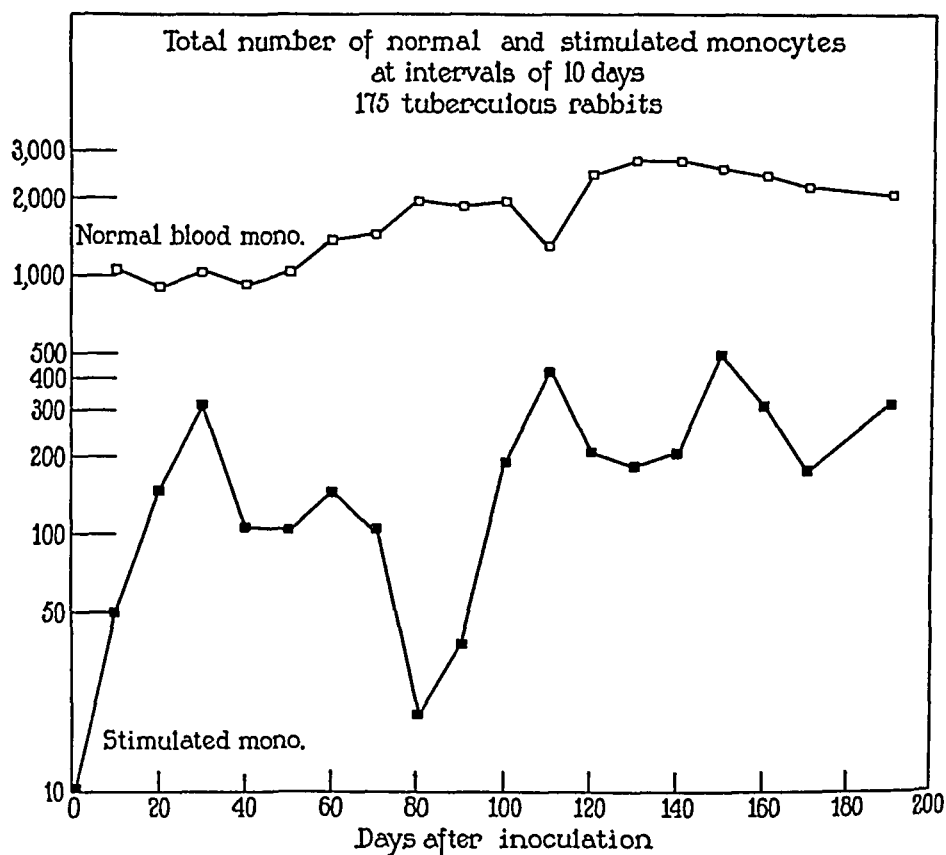


FIG. 7. The relative number of normal and stimulated monocytes in the blood during successive 10 day periods after inoculation, in the same group of 175 tuberculous rabbits.

Fig. 3), whereas at other times when there are no stimulated forms present epithelioids are not seen. The surface film and the mitochondria of the two types of monocyte are similar. These qualitative differences between cells of the monocytic strain are only to be seen with the supravital technique. Fig. 7 shows the total number of normal monocytes and stimulated monocytes plotted separately,

instead of together as in Fig. 6. It is clear that the small rise in the total number of monocytes which occurred in the 1st month was due to the accession of several hundred of the stimulated forms per c.mm. The normal monocytes remained at their initial level until the 2nd month, when they rose steadily. The appearance of stimulated monocytes in the peripheral blood is a constant phenomenon following inoculation with tuberculosis, particularly in the 1st month (8). After this time these stimulated cells decrease somewhat in number and frequency, reappearing during periods of great extension of lesions.

The basophilic leucocytes and the eosinophils reacted as did the neutrophils, showing a leucopenia at the end of the 1st month. Eosinophils became exceedingly scarce, the average number per c.mm. at the end of the 1st month being but 35. Both eosinophils and basophils returned to approximately normal levels after the 1st month.

It is seen in Fig. 6 that the neutrophils after the period of leucopenia returned to their former level and went on to a definite leucocytosis, which was maintained throughout the course of the disease in most instances. This leucocytosis was probably a reaction to the presence of caseous lesions. Caseation occurred so constantly that it is difficult to determine what other factors, if any, contributed to the increase in neutrophils.

Thus the complex of lesions during the first phase, at first extending and then regressing in some organs, while steadily progressing in others, can be viewed not only with the aid of the autopsy findings, but followed from day to day and from week to week in the living animals, by means of repeated studies of the blood cells.

In the chronic phase of the disease, the lesions were for the most part confined to the lungs and to the kidneys. The other organs showed minimal lesions. It was also evident that the rate of extension of the lesions was not so great in the chronic phase, since many animals showed no evidence of any change in their clinical condition for long periods of time and died with relatively few lesions. During this period of relative inactivity, studies of the blood cells showed few significant changes. However, in each case in which the studies were made at frequent intervals up to the time of death, it was found that for a period of 1 to 2 months before death there was a change in the blood picture, of a constant nature. The lymphocytes, which had



been maintained at or near the normal level, about 3,500, during a long period of inactivity, started to fall; this fall was steady and continued until the total numbers of lymphocytes just before death were but 600 to 1,200 cells. During this period of decline the monocytes frequently increased in total number and stimulated forms became more frequent. The animals' clinical condition went steadily downward, the weight falling at about the same rate as the lymphocytes. This decrease in the number of lymphocytes and increase in the number of monocytes at a time when the animals' resistance to the disease has obviously failed is a change that occurs so constantly that it is extremely useful as an index or basis for prognosis.

It will be noted that no mention has been made of a relation between the extent of the lesions and the longevity in the chronic phase. The number of animals that have been studied is not large enough to draw conclusions from, and it is not clear that such a relation exists at least as measured by the extent of the cellular changes. The fact that many animals of extraordinary resistance, surviving for a year or more, show at autopsy only a few lesions, most of which are well walled in with fibrous tissue, would suggest that the extent of the lesions in the chronic phase rose and fell somewhat parallel to the death rate, as in the acute phase. However, when the group of animals in the chronic phase which showed the most extensive lesions were compared with those which showed the least, it was found by analyzing the blood studies and the weight records that the two groups showed the same prolonged period of decline and wasting before death. It would appear that the factors responsible for the loss of weight and malnutrition are in part independent of the extent of the lesions. The close correlation between the maintenance of weight and the maintenance of the lymphocytes, and the simultaneous decline in both suggests that the lymphocytes are either directly or indirectly related to the state of resistance to the disease.

#### *The Relation of the Preinoculation Blood Counts in Rabbits to Their Resistance to Tuberculosis*

The records of 66 animals, all of which were inoculated with 0.1 mg. of tubercle bacilli and which had an average of 5.4 counts each before inoculation, were examined in an effort to find what relationships

could be discovered between the blood counts before infection and the resistance of the animal. The counts on each animal were summed and the mean obtained. The means for the group of 66 animals were then arranged in a frequency distribution and the mean, mode, and standard deviation calculated. It was found that the separate values distributed themselves in the unimodal fashion of a normal frequency distribution. A correlation table was then made for each of the various cell types, plotting the longevity of the animal as the ordinate and fractional units of standard deviation on either side of the mode along the abscissa. It was found that a positive correlation existed between longevity and blood counts of or near to the modal value. Those animals of exceptional longevity had counts which deviated but slightly from the mode, while conversely those animals whose counts varied significantly from the mode were found to have survived but a very limited time. Such relationships have been shown by Casey and Pearce (9) to hold in regard to the susceptibility of rabbits to a malignant neoplasm. This correlation interestingly enough was evident when the whole group of animals was considered. However, when the animals were grouped according to whether or not they survived less than or longer than 90 days, it was seen that the correlation was slight in the first group and very definite in the group surviving beyond 90 days. This indicates apparently that some factors other than those expressed in the circulating blood cells are responsible for the division of any group into those which will survive and those which will not survive the first phase of the disease, since there was no significant difference in the blood counts of the first group and the second. The total number of animals used was not large enough, however, for such an analysis, and it may well be that with additional data the conclusions will be modified or amplified.

The relation between longevity and the value of the deviation of the count was most evident in the red blood cells; among the white cells the lymphocytes and basophils showed a closer correlation than did the neutrophils and monocytes. No direct relationship between longevity and the preinfection ratio of lymphocytes to monocytes could be demonstrated, a very high ratio apparently being as frequently associated with low longevity as a low ratio.

been maintained at or near the normal level, about 3,500, during the long period of inactivity, started to fall; this fall was steady and continued until the total numbers of lymphocytes just before death were but 600 to 1,200 cells. During this period of decline the monocytes frequently increased in total number and stimulated forms became more frequent. The animals' clinical condition went steadily downward, the weight falling at about the same rate as the lymphocytes. This decrease in the number of lymphocytes and increase in the number of monocytes at a time when the animals' resistance to the disease has obviously failed is a change that occurs so constantly that it is extremely useful as an index or basis for prognosis.

It will be noted that no mention has been made of a relation between the extent of the lesions and the longevity in the chronic phase. The number of animals that have been studied is not large enough to draw conclusions from, and it is not clear that such a relation exists at least as measured by the extent of the cellular changes. The fact that many animals of extraordinary resistance, surviving for a year or more, show at autopsy only a few lesions, most of which are very well walled in with fibrous tissue, would suggest that the extent of the lesions in the chronic phase rose and fell somewhat parallel to the death rate, as in the acute phase. However, when the group of animals in the chronic phase which showed the most extensive lesions were compared with those which showed the least, it was found on analyzing the blood studies and the weight records that the two groups showed the same prolonged period of decline and wasting before death. It would appear that the factors responsible for the loss in weight and malnutrition are in part independent of the extent of the lesions. The close correlation between the maintenance of weight and the maintenance of the lymphocytes, and the simultaneous decline in both suggests that the lymphocytes are either directly or indirectly related to the state of resistance to the disease.

#### *The Relation of the Preinoculation Blood Counts in Rabbits to Their Resistance to Tuberculosis*

The records of 66 animals, all of which were inoculated with 0.1 mg of tubercle bacilli and which had an average of 5.4 counts each before inoculation, were examined in an effort to find what relationships

could be discovered between the blood counts before infection and the resistance of the animal. The counts on each animal were summated and the mean obtained. The means for the group of 66 animals were then arranged in a frequency distribution and the mean, mode, and standard deviation calculated. It was found that the separate values distributed themselves in the unimodal fashion of a normal frequency distribution. A correlation table was then made for each of the various cell types, plotting the longevity of the animal as the ordinate and fractional units of standard deviation on either side of the mode along the abscissa. It was found that a positive correlation existed between longevity and blood counts of or near to the modal value. Those animals of exceptional longevity had counts which deviated but slightly from the mode, while conversely those animals whose counts varied significantly from the mode were found to have survived but a very limited time. Such relationships have been shown by Casey and Pearce (9) to hold in regard to the susceptibility of rabbits to a malignant neoplasm. This correlation interestingly enough was evident when the whole group of animals was considered. However, when the animals were grouped according to whether or not they survived less than or longer than 90 days, it was seen that the correlation was slight in the first group and very definite in the group surviving beyond 90 days. This indicates apparently that some factors other than those expressed in the circulating blood cells are responsible for the division of any group into those which will survive and those which will not survive the first phase of the disease, since there was no significant difference in the blood counts of the first group and the second. The total number of animals used was not large enough, however, for such an analysis, and it may well be that with additional data the conclusions will be modified or amplified.

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# THE RÔLE OF HYPERSENSITIVITY IN THE PRODUCTION OF EXPERIMENTAL MENINGITIS

## I. EXPERIMENTAL MENINGITIS IN TUBERCULOUS ANIMALS

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PLATE 10

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The hypersensitive state may be of significance in the pathogenesis of certain central nervous system disturbances. Of primary importance is the possible relationship of the postexanthematous and post-vaccinal encephalitides to an allergic state of the central nervous system. Glanzmann (1) and Rivers (2) emphasize the hypersensitive state as a rôle in the nervous complications of chicken-pox, smallpox, measles and vaccination. Rackemann (3) suggests that certain of the migrains may be due to the patient's idiosyncrasy to some foreign protein. A review of cases of epileptics, whose attacks were relieved when food and other products to which they were found hypersensitive were removed from their diets and environment respectively, will be found in the monograph on epilepsy by Lennox and Cobb (4). The relationship between allergy and the severity of tuberculous meningitis will be referred to later. It was because of the suggested association of allergy to the above mentioned nervous maladies that the following experiments were designed. We wished to study the potentialities of the central nervous system in sensitized animals to react when brought in contact with the homologous antigen.

Tuberculous animals were first employed because it is known that their tissues are sensitive to tuberculin. The subarachnoid space was chosen as the locus of study because of its easy accessibility through the basal cistern and, furthermore, because of its very intimate relationship with the brain parenchyma. The direct extension of the subarachnoid space around the blood vessels into the depth of the brain parenchyma to envelop the individual nerve cells in all probability, is

## SUMMARY

It has been shown that in a large group of rabbits inoculated intravenously with bovine tubercle bacilli the disease which follows resolves itself into two distinct phases. The first phase manifests itself in widespread diffuse lesions which subsequently regress. The mortality rate shows a rise and fall during this period, which have been correlated with the extent of lesions and with the changes in the blood cells. The duration of this phase is approximately 80 to 90 days. Following this period the mortality rate again rises and falls, during a period when all animals show lesions of a chronic nature. In an infected group the number of fatalities in the first phase is a function of the size of the dose. When the size of the dose and other conditions are held constant, a definite basis upon which to compare the reactions of treated animals is established.

Studies of the blood cells during the course of the disease show that the changes in these cells reflect the course of the lesions in the first phase when other signs are lacking and offer a valuable means of making an estimate of the state of resistance of the animal from time to time.

Studies of blood counts made on rabbits before their inoculation with tuberculosis were analyzed with regard to the relative longevity of the animals. It was found that those animals whose blood cells of various sorts deviated least from the modal value for the entire group survived longer than those animals whose counts were significantly high or low.

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several animals with far advanced tuberculosis were sacrificed without having received any injection in the basal cistern. Histological sections were prepared as described above. Many non-tuberculous guinea pigs were likewise inoculated *via* basal cisterns with old tuberculin and glycerine broth.

### RESULTS

Several preliminary experiments were devised in order to determine the nature of the clinical response and the type of lesion produced in the central nervous system by the inoculation of tuberculin into tuberculous animals. The results were constant both clinically and pathologically.

Within 4 to 5 hours after the inoculation of tuberculin into the cisterns, restlessness and ruffling of hair appeared which became progressively more severe until twitchings and convulsions resulted. Marked weakness, incontinence and opisthotonus were followed shortly by death which occurred within 6 to 12 hours. The tuberculous animals receiving glycerine broth as well as the non-tuberculous animals which received tuberculin never showed any clinical evidence of cerebral irritation.

When histological sections of the brains were prepared from tuberculous guinea pigs inoculated with tuberculin, there was found in the subarachnoid space, an extensive exudate which extended over the convexities of the brain and into the sulci. In many instances there was a subpial as well as a marked cortical perivascular infiltration. There was no demonstrable evidence of damage to the cells of the brain parenchyma. The control animals showed either a very slight non-specific reaction or none at all. Tuberculous animals that received the glycerine broth *via* the cisterns, always responded histologically by a very slight non-specific type of reaction.

After having established the response in a hypersensitive animal, primarily in the leptomeninges of the central nervous system, an experiment was devised to determine the period of maximum response and to correlate the clinical manifestations with the histological findings as shown in Table I. The animals used for this experiment had far advanced generalized tuberculosis. The quantity of tuberculin inoculated *via* basal cisterns was 0.3 cc. of a 1/100 dilution in physiological saline.

The results demonstrate that in guinea pigs showing extensive generalized tuberculosis there rapidly develop definite clinical symptoms that are apparently associated with the anatomical response of



known from the work of Weed (5) and Cushing (6). It is impossible, in our judgment, to separate the subarachnoid space as a space in which spinal fluid bathes the surface of the brain from its extension to the very nerve elements of the brain proper. Such being the case, any reaction which can be made to occur in the fluid spaces covering the brain surface, will, in all probability, result likewise in the deeper portions of the brain substance under proper experimental conditions.

### *Methods*

Guinea pigs averaging 200 to 300 gm. were inoculated subcutaneously in the groin with a light suspension of freshly isolated virulent human tubercle bacilli. The tubercle bacilli were grown, either on glycerine agar slants or Dorset's egg media. The tuberculosis in the animals was permitted to progress to various stages before the test inoculation was made. Old tuberculin was diluted in physiological saline to the concentration desired. Not more than 0.2 to 0.3 cc. of each dilution could be safely inoculated into the basal cisterns of the animals. A small hypodermic needle, size No. 26, measuring  $\frac{3}{8}$  inch in length was used. It was unnecessary to anesthetize the animals for the procedure. The immediate pressure symptoms that developed after the inoculation indicated that the injection mass had entered the cisterns. These symptoms disappeared within a few seconds with complete recovery. The point of inoculation into the basal cisterns was determined by finding the crest of the occipital bone at the midline and puncturing the skin 1 cm. below this point. The needle was then permitted to strike the base of the occipital bone in the midline and forced gently downward until the rim of the foramen magnum was reached. At this point the needle perforated the occipital-atlantoid ligament and the dura with ease. Extreme care was taken to see that the needle did not penetrate too far so as to cause trauma to the brain stem. The material was slowly injected. Observations were frequently made for any clinical symptoms that could be attributed to the traumatic effects of the material inoculated into the subarachnoid space. If there was no change observed, the animals were permitted to live for various lengths of time. The animals that did not die were killed (under complete ether anesthesia) by means of decapitation. They were immediately autopsied. Both gross and microscopic studies were made of the viscera in order to determine the extent of the tuberculosis. The brain was removed, sectioned and fixed immediately in 95 per cent alcohol and stained with iron-hematoxylin and eosin and Nissl techniques. Bacteriological methods were employed in aseptically removed portions of the brain to rule out presence of either the ordinary bacteria or the tubercle bacillus. The inoculation of tuberculin into the carotid arteries was performed under ether anesthesia.

Every experiment included controls of both the tuberculous and non-tuberculous animals. The tuberculous animals had glycerine broth equivalent to the concentration and dosage found in old tuberculin inoculated into the basal cistern. Also,

TABLE II

Interval between initial inoculation and tuberculin inoculation	Result of skin test	Clinical symptoms	Extent of visceral tuberculosis	Lesion of central nervous system
days				
5	Neg.	None	No gross or microscopic evidence of tuberculosis	Slight but definite exudate at base of brain More than a non-specific response
7	"	"	" "	" "
10	Pos.	Restlessness	Inguinal lymph nodes enlarged; few microscopic tubercles in spleen and lungs	Moderate amount of exudate in sulci and over convexities of brain with perivascular infiltration
12	"	Marked restlessness	Same as for 10 day period	Moderate amount of exudate in sulci and over convexities of brain with subpial infiltration
14	"	Marked restlessness with progressive weakness	Enlarged inguinal lymph nodes with necrosis; microscopic tubercles in spleen and liver	Extensive exudate in sulci and diffuse over convexities of brain with subpial and perivascular infiltration
19	"	Twitching of muscles, restlessness and progressive weakness. Loss of sphincter control	Inguinal lymph nodes and spleen enlarged and nodular; microscopic tubercles in lung and spleen	Extensive exudate in sulci and over convexities of brain with perivascular infiltration
24	"	Onset of twitching of muscles within 5 hrs. Convulsions and death within 6 hrs.	Inguinal lymph nodes enlarged and necrotic; gross evidence of tubercles in lungs and spleen with microscopic tubercles in liver	Extensive exudate in sulci and over convexities of brain; subpial and perivascular infiltration very distinct

the leptomeninges. Histological changes were not demonstrable until 4 hours after the inoculation of the tuberculin. The maximum response occurred within 8 to 12 hours. There were slight variations noted in the quantity of exudate in the meninges, but this may be attributed to the differences in response of the individual animal, as well as upon the variation of the amount of visceral tuberculosis present.

Since guinea pigs with far advanced generalized tuberculosis uniformly developed the most widespread exudate in the subarachnoid space within 8 to 12 hours, it was possible to take this time as a maximum period after the inoculation of tuberculin for sacrificing the animals. An experiment was devised to study the relation between

TABLE I

Total No. of animals	Survival time after tuberculin inoculation	Clinical symptoms	Lesions of the central nervous system
	<i>hrs.</i>		
4	2	None	No lesions
4	4	"	Definite polymorphonuclear exudate in sulci and over convexities of brain
4	6	Definite progressive weakness and twitch- ings	Extensive polymorphonuclear exudate over convexities with marked subpial and perivascular infiltration
8	8-12	Death of all animals	" "

the amount of visceral tuberculosis and the severity of the hyper-sensitive response in the leptomeninges.

A series of guinea pigs were inoculated subcutaneously into the groin with a virulent strain of human tubercle bacilli. At designated intervals (Table II) two guinea pigs were selected from the group and each inoculated with 0.3 cc. of a 1/100 dilution of tuberculin in physiological saline. If the animals did not develop symptoms and die before 12 hours, they were sacrificed. A skin sensitivity test was performed 24 hours before the inoculation of the tuberculin into the basal cistern. Bacteriological studies, both for the presence of tubercle bacillus and for aerobic and anaerobic bacteria were made with portions of the brain tissue.

Non-tuberculous animals inoculated in the basal cistern with tuberculin and tuberculous animals inoculated with glycerine broth were used as controls. Some

TABLE II

Interval between initial inoculation and tuberculin inoculation	Result of skin test	Clinical symptoms	Extent of visceral tuberculosis	Lesion of central nervous system
days				
5	Neg.	None	No gross or microscopic evidence of tuberculosis	Slight but definite exudate at base of brain More than a non-specific response
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10	Pos.	Restlessness	Inguinal lymph nodes enlarged; few microscopic tubercles in spleen and lungs	Moderate amount of exudate in sulci and over convexities of brain with perivascular infiltration
12	"	Marked restlessness	Same as for 10 day period	Moderate amount of exudate in sulci and over convexities of brain with subpial infiltration
14	"	Marked restlessness with progressive weakness	Enlarged inguinal lymph nodes with necrosis; microscopic tubercles in spleen and liver	Extensive exudate in sulci and diffuse over convexities of brain with subpial and perivascular infiltration
19	"	Twitching of muscles, restlessness and progressive weakness. Loss of sphincter control	Inguinal lymph nodes and spleen enlarged and nodular; microscopic tubercles in lung and spleen	Extensive exudate in sulci and over convexities of brain with perivascular infiltration
24	"	Onset of twitching of muscles within 5 hrs. Convulsions and death within 6 hrs.	Inguinal lymph nodes enlarged and necrotic; gross evidence of tubercles in lungs and spleen with microscopic tubercles in liver	Extensive exudate in sulci and over convexities of brain; subpial and perivascular infiltration very distinct

TABLE II—*Concluded*

Interval between initial inoculation and tuberculin inoculation	Result of skin test	Clinical symptoms	Extent of visceral tuberculosis	Lesion of central nervous system
<i>days</i>				
25	—	Rapid onset of twitchings and convulsions resulting in death within 5½ hrs.	Inguinal lymph nodes enlarged and caseous; gross evidence of tubercles in lungs and spleen; microscopic tubercles in liver	Extensive exudate in sulci and over convexities of brain; marked subpial and perivascular infiltration
31	—	Rapid onset of weakness and convulsions. Loss of control of sphincters. Death within 9 hrs.	Gross evidence of tubercles in lungs, spleen and liver; inguinal lymph nodes are caseous	Extensive exudate in sulci and over convexities of brain
37	Pos.	Rapid onset of weakness and death within 6 hrs.	Extensive generalized tuberculosis throughout organs	Extensive exudate in sulci and over convexities of brain; marked subpial and perivascular infiltration

of the tuberculous animals were inoculated with tuberculin into the neck muscles, and others that received no injection of tuberculin were sacrificed from time to time. These controls never showed any clinical symptoms nor was there any evidence of anatomical changes in the subarachnoid spaces comparable to those found in the experimental animals. The tuberculous animals that received glycerine broth showed a slight non-specific response characterized by a polymorphonuclear exudate.

These results indicate that the degree of the response of the central nervous system tissues to tuberculin is directly related to the extent of the visceral tuberculous disease.

The following two experiments (Experiments III and IV) were devised to determine the effects of high dilutions of tuberculin upon the leptomeninges and to study the transformation in the exudate produced within the leptomeninges.

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TABLE III

Duration of infection	No. of animal	Survival time after tuberculin inoculation	Clinical symptoms	Extent of visceral tuberculosis	Lesions of the central nervous system
wks.		hrs.			
1	1	D 24	None	Very slight inguinal lymph node enlargement. No microscopic evidence of tubercles	No exudate or change observed
	2	K 48	"		
	3	K 120	"		
	4	K 120	"		
3	5	K 24	Every animal showed slight restlessness, twitchings and ruffled hair	Definite enlargement of inguinal lymph nodes. Microscopic evidence of tubercles in liver, spleen and lungs	Slight exudate in sulci consisting of equal proportions of lymphocytic and polymorphonuclear cells
	6	K 48			
	7	D 96			
	8	K 120			
5	9	D 8	Marked restlessness, twitchings and weakness	Inguinal lymph nodes enlarged; extensive tubercles throughout lungs, liver and spleen	Extensive exudate in sulci and over convexities; polymorphonuclear and few lymphocyte cells
	10	K 24			
	11	D 5½			
	12	D 5½			
			Progressive and rapid development of symptoms and death	Inguinal lymph node enlargement; tubercles in liver, spleen and lungs	No exudate in meninges
			" "	Extensive generalized tuberculosis	No exudate
			" "	" "	Extensive polymorphonuclear exudate in sulci and over convexities with perivascular infiltration

D = dead. K = killed.

The tuberculous animals were chosen at intervals after the initial infection and inoculated with 0.3 cc. of a 1/10,000 dilution of tuberculin in physiological saline. The animals surviving for 120 hours were then sacrificed.

In addition to the usual controls, two others were used. One animal at the 1 week period was inoculated with a 1/10 dilution of tuberculin. It had no clinical symptoms and very slight polymorphonuclear exudate at the base of the brain. The other animal was chosen at the 3 week period and also inoculated with a 1/10 dilution resulting in the death of the animal within 12 hours and a very extensive polymorphonuclear exudate in the leptomeninges.

The results of the experiment are definite as concerns the clinical findings and pathological picture.

As the tuberculous process advanced within the viscera of the guinea pigs, there was a rapidly increasing susceptibility of the leptomeninges to the tuberculin. The central nervous system showed a quantitatively less and qualitatively different type of reaction from the tuberculous guinea pigs inoculated with more concentrated dilutions of tuberculin. When the animals were killed or died within 24 to 48 hours, the exudate was limited chiefly to the base of the brain and Sylvian fissures. The exudate usually consisted of equal proportions of the polymorphonuclear and lymphocytic cells. If the guinea pigs were not sacrificed or did not die before 120 hours there was observed either a very slight amount of residual exudate composed chiefly of small lymphocytic cells or the brain showed no anatomical changes. An occasional guinea pig, however, especially in this experiment, would manifest definite clinical symptoms as intense and typical as those previously described, but upon examination of the sections from the brain, there was no exudate present or any other demonstrable lesion. This phenomenon was noticed twice in the experiments in which greater concentrations of tuberculin (1/10 and 1/100) were inoculated into the basal cisterns. These guinea pigs at the time of inoculation of the material into the basal cistern showed very definite immediate pressure symptoms with the usual rapid recovery which indicated that the material reached the basal cistern. A possible explanation is that a few guinea pigs from a group are refractory to reacting in an acute manner; in other words, a state of anergy is present.

The following experiment was likewise performed to produce an exudate within the subarachnoid space as well as to permit the animal to survive.

Guinea pigs were used that showed a stage of the tuberculosis in which the lymph nodes and spleen were definitely enlarged, but the other organs in the gross did not show the presence of tubercles; in other words, a tuberculous infection was established which had not become generalized. One of two dilutions of tuberculin (1/100 and 1/1000) was inoculated into the basal cistern. If the animals did not die at the time desired, they were sacrificed.

TABLE IV

Dilution of tuberculin	No. of animal	Survival time after tuberculin inoculation	Clinical symptoms	Extent of visceral tuberculosis	Lesions of the central nervous system
1/100	1	hrs. K 12	Gradual weakness, restlessness and twitchings of muscles	Inguinal lymph nodes and enlarged caseous tubercles in spleen; microscopic tubercles in lungs	Moderate amount of polymorphonuclear exudate in sulci and over convexities. Some perivascular infiltration
		2 D 36	Few hrs. before death, developed twitching of muscles, paralysis of hind legs and weakness	" "	Extensive polymorphonuclear exudate and a few mononuclear cells in sulci and over convexities
		3 D 60	Few hrs. before death, developed twitchings and extreme weakness	" "	Moderate lymphocytic exudate at base of brain with slight amount over convexities
1/1000	4	D 6	Gradual loss of power in hind legs, weakness and death	Inguinal lymph nodes and enlarged caseous tubercles in spleen	Extensive polymorphonuclear exudate with subpial and perivascular infiltration
		5 K 60	Ruffling of hair	Inguinal lymph nodes and enlarged caseous. Spleen and liver show gross tubercles. Microscopic tubercles in lungs	Moderate lymphocytic exudate at base of brain, and slight amount over convexities
		6 D 120	Gradual loss of weight, progressive weakness, death	Generalized tubercles throughout viscera	" "

D = dead. K = killed.



The results of the experiment show the variation noted in the previous experiments.

The animals that were killed or died within the 6 to 12 hour period, responded with an exudate that extended over the convexities of the brain as well as showing marked perivascular infiltration. The exudate consisted entirely of polymorphonuclear and large mononuclear cells. The guinea pigs that survived from 12 to 120 hours, showed an exudate which was limited chiefly at the base of the brain and appeared slightly over the convexities. But, instead of a polymorphonuclear cellular response such as was found consistently in the guinea pigs dying early, there was a lymphocytic cell present. The cause of death of the animals could not be correlated with the exudate found in the subarachnoid space. It is known that when an exudate, not associated with necrosis, resolves, there is a gradual decrease of polymorphonuclear cells of the exudate and there is to be seen a mononuclear type of cell, the lymphocyte. This in turn gradually disappears so that the organ is returned to its apparently initial state. Since there is no evidence of necrosis in this response of the meninges, the exudate found in the subarachnoid space of the animals that survived for several days was probably the result of a gradual resolution of the more acute process that was previously elicited.

Thus far all the experiments have been limited to the inoculation of material into the subarachnoid space, which brought about the vigorous response in the leptomeninges. The cells of the brain parenchyma showed no evidence of damage by the technique used for this study. In order to bring the tuberculin into closer contact with the ganglion cells of the cortex, the skull was trephined while the animal was under ether anesthesia, the needle inserted directly into the cortex and the material injected. A dilution of tuberculin 1/100 was inoculated. The results are presented in Table V.

Instead of damage to the surrounding cells of the brain, there developed extensive generalized meningitis with marked perivascular infiltration. All the animals died within 6 to 8 hours after the inoculation. This response was similar to the reaction obtained when the material was inoculated by the basal cistern.

Carotid injections of tuberculin were done in guinea pigs with far advanced tuberculosis. The results were uniformly negative in the few animals observed. This phase of the problem is being studied.

Controls of both tuberculous and non-tuberculous animals were employed for every experiment. A summary of the controls is given in Table VI.

The complete lack of clinical symptoms and the absence of path-

TABLE V

No. of animal	Clinical symptoms	Extent of visceral tuberculosis		Lesions in central nervous system
		Extensive general	ized tuberculosis	
1	Restlessness, twitchings, weakness and death in 8 hrs.			Moderate polymorphonuclear exudate in sulci and over convexities with perivascular infiltration
2	Restlessness, twitchings, weakness and death in 6 hrs.	"	"	Extensive polymorphonuclear exudate in sulci and over convexities with perivascular infiltration
3	" "	"	"	Extensive polymorphonuclear exudate in sulci and over convexities. Hemorrhage along the needle tract
4	None	None		Very slight polymorphonuclear and lymphocytic exudate in sulci

TABLE VI

TABLE VI					
No. of animals	Type of material inoculated into cisterna	Quantity inoculated	Clinical symptoms	Lesions in viscera	Lesions of the central nervous system
Tuberculous animals					
22	Glycerine broth	0.3	None	Extensive visceral tuberculosis	Very slight polymorphonuclear exudate
4	Tuberculin into muscles of neck and peritoneal injection	0.3	"	" "	No reaction
3	No inoculation	—	"	" "	" "
Non-tuberculous animals					
12	Glycerine broth	0.3	None	None	No reaction
23	Tuberculin	0.3	"	"	" "
4	No inoculation	—	"	"	" "

ological changes in the central nervous system is in strong contrast with the findings in the hypersensitive animals. All of the control

tuberculous animals showed a very slight, non-specific, polymorphonuclear exudate in response to the glycerine broth inoculated into the basal cistern. None of the controls died from the inoculation.

Both living and dead tubercle bacilli were next tested to determine the response of the leptomeninges in hypersensitive animals.

In the case of living tubercle bacilli suspensions of two concentrations were used. One consisted of a concentration of tubercle bacilli that gave an opalescent appearance while the other suspension consisted of a concentration of tubercle bacilli

TABLE VII

No. of animals used	Clinical symptoms and survival period	Extent of tuberculosis in viscera	Lesions of the central nervous system
3	Rapid progressive symptoms of weakness, twitchings and convulsions. Death in 3 to 5 hrs.	Enlarged inguinal lymph node. Spleen enlarged and nodular. Few tubercles in liver and lungs	Extensive polymorphonuclear exudate in sulci and over convexities of brain with marked perivascular infiltration
1	Ruffling of hair, anorexia, weakness of hind legs, death within 10 days	Enlarged caseous inguinal lymph nodes. Liver and lungs show many tubercles	Exudative and proliferative meningitis. Tubercle formation in perivascular spaces and parenchyma. Thrombi in vessels of brain
Controls			
4	No reaction. Killed at 24 hrs.	None	Very slight non-specific polymorphonuclear exudate deep in the sulci

that contained not over 250 bacilli per high power field. In Table VII the effects of the concentrated suspension of tubercle bacilli in allergic animals are shown.

The concentrated living tubercle bacilli were capable of eliciting a very vigorous response in the leptomeninges of a hypersensitive animal. The animal that survived for a period of 10 days showed an exudate and proliferative type of response. There were many tubercles throughout the perivascular spaces of the brain.

Table VIII demonstrates the results of inoculating minute quantities of living tubercle bacilli into the basal cistern of tuberculous animals.

The number of days that the animal survived after the inoculation of the organisms is noted.

In comparing the lesions in the central nervous system of the animals that have far advanced visceral tuberculosis with those of the non-tuberculous animals, there is observed the absence of tubercles

TABLE VIII

TABLE VIII			
No. of animal	Survival period after cisterna inoculation	Extent of visceral tuberculosis	Lesions found in central nervous system
250 tubercle bacilli per high power field			
1	2	Extensive generalized tuberculosis	Slight amount of lymphocytic infiltration in meninges
2	9	Moderate generalized tuberculosis	Very slight lymphocytic response of the meninges
3	23	Extensive generalized tuberculosis	Slight proliferative and lymphocytic exudate in the meninges
4	27	" "	" "
5	29	" "	Moderate degree of proliferative and exudative response. Exudate predominantly lymphocytic. Occasional tubercle in cortex
6	29	" "	" "
Controls (non-tuberculous)			
7	30	Lymphoid hyperplasia of spleen	Extensive proliferative and exudative response. Many tubercles in meninges and throughout cortex
8	20	" "	" "
9	25	" "	" "
10	28	" "	" "
11	29	Dead—pneumonia	No evidence of change
12	2		

in the former group of guinea pigs. The very slight lymphocytic response in the leptomeninges is the only evidence of change in the central nervous system, while the control animals revealed marked evidence of proliferation and tubercle formation throughout the cortex and meninges. Even the tuberculous animals that lived as long as the controls showed only an occasional tubercle in the brain. Soper and

Dworski (7) have clearly demonstrated in their experiments of superinfection in the meninges of rabbits this difference between tuberculous and non-tuberculous animals. Dead tubercle bacilli were studied as to the type of response that would be elicited in the hypersensitive animal. A portion of the same suspension prepared for the experiment used in studying the effects of large concentrations of whole tubercle bacilli was heated in a water bath at 60°C. for 1 hour. The viability of the culture was tested by inoculation of the heated

TABLE IX

No. of animals used-	Clinical symptoms and survival period	Extent of visceral tuberculosis	Lesions of central nervous system
3	Ruffling of hair, twitchings, convulsions and death within 4 to 12 hrs.	Extensive generalized tuberculosis throughout the viscera	Extensive polymorphonuclear exudate in sulci and over convexities of brain. Marked subpial and perivascular infiltration
Controls			
2	No clinical symptoms. One animal sacrificed within 12 hrs.; the other permitted to live for 48 hrs.	None	Slight polymorphonuclear exudate at the base of the brain
1	Not inoculated	Extensive generalized tuberculosis	No exudate or change in the central nervous system

culture into normal guinea pigs. There was no evidence of tuberculosis 2 months after the initial inoculation.

Dead tubercle bacilli are capable of eliciting a response of the meninges in allergic animals.

#### DISCUSSION

In comparing the response of the leptomeninges in these experiments with the allergic response in other organs of tuberculous animals our results are in general those one might expect to find. The sterile meningitis produced in animals allergic to tuberculin is similar in its fundamentals to the tuberculin reaction of the skin in hypersensitive

animals. The seat of the response lies in the tissue spaces of the leptomeninges instead of the subcutaneous tissues. Similar allergic states have been produced in other organs of the tuberculous animals. Austrian and Willis (8), Soper and coworkers (9) and more recently Larson and Long (10) have produced pneumonia in tuberculous animals by bringing tuberculin in contact with the lungs. Long and Finner (11) and Long and coworkers (12) produced an acute glomerular nephritis in tuberculous animals by injecting tuberculin in particular state into the renal artery. Long (13) has demonstrated marked changes in the testis of tuberculous animals due to tuberculin inoculations.

No apparent differences exist between the response that occurs in the lungs, kidneys and testis and the one that occurs in the meninges. The response is characterized by an exudate that is composed chiefly of polymorphonuclear cells. It is elicited within 6 to 12 hours after the inoculation of the tuberculin. The death of the animal may result, depending upon the location of this response. If the animal survives, the recovery takes place by resolution or by organization, depending upon whether there is necrosis present. Necrosis in the meninges, however, was not common. The resolving exudate consists chiefly of small lymphocytic cells.

When tuberculin was inoculated directly into the carotid artery of allergic animals there was no response elicited in the meninges of the hypersensitive animals. This lack of response is attributed to the failure of the antigen to remain localized for sufficient length of time and also to the dilution of the tuberculin. An attempt to demonstrate the presence of tuberculin in the spinal fluid of one of these animals failed. This failure may be due to technique; further attempts to demonstrate it are necessary. If sufficiently large quantities of tuberculin can be given without bringing about the death of the animal, it may be possible to establish the response in the central nervous system. Or, if the tuberculin is permitted to remain localized within the brain substance by means of embolic particulate matter, the allergic response will undoubtedly result.

Unequal intensity and rapidity of response of the organs when the body as a whole is sensitized to a foreign protein is a possibility. Stewart (14) found that the tuberculin reaction became positive on the

5th day in the testicle which was the primary focus of infection, and did not appear in the opposite organ until later. The leptomeninges of the tuberculous guinea pig responded within 5 days by a definite specific reaction, while the skin manifestations did not appear before the 10th day. However, the meninges were not the focus of primary infection. It is possible that the early response of the meninges is due to the anatomical structure of the organ and exudate, being more readily demonstrable in the earliest stage.

Glycerine broth always elicited a non-specific exudative response in the leptomeninges of tuberculous animals, while an equivalent quantity of glycerine broth administered to non-tuberculous animals yielded a very slight exudative response or none. Friedberger and Gajzago (15), Borrel (16), Somerfeld and Ziskin (17) found that tuberculous animals responded more vigorously to non-specific proteins than did the non-tuberculous animals.

Both dead and living tubercle bacilli when inoculated in sufficient quantities *via* the basal cistern of tuberculous animals caused an acute and vigorous response which terminated in the death of the animal, while smaller quantities of living tubercle bacilli resulted in a less severe acute reaction and a greater portion of the animals survived for a longer period of time. Histological sections of the brains obtained from the tuberculous animals that had living tubercle bacilli inoculated into the basal cisterns showed only an occasional tubercle and a moderate number of small lymphocytes at the base of the brain. On the other hand, the non-tuberculous animals that received small quantities of living tubercle bacilli had an extensive proliferative and exudative tuberculous response throughout the meninges and brain substance. Soper (7) has shown that the introduction of tubercle bacilli into an already tuberculous subarachnoid space will produce a sudden exacerbation of meningeal signs in the animals. There is an associated increase in cell count of the spinal fluid. The primary inoculations of non-tuberculous animals *via* the leptomeninges does not result in an acute onset of meningeal signs, but leads to a slow progressive type of tuberculous meningitis. He demonstrated that the superinfected animals did not reveal so extensive a type of meningitis when compared with the control animals. The primary focus in Soper's experiments was in the central nervous system, while the

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primary focus in our animals was established in the viscera by inoculation into the groin. Both Austrian (18) and Borrel (16) found that the injection of tuberculin into the subarachnoid spaces of an animal with tuberculous meningitis during the latent period aggravated the symptoms and produced a more rapidly fatal termination of the animal's life.

Clinical observations of tuberculous meningitis have been reported in the literature in which the injection of tuberculin into the subarachnoid spaces has resulted in a definite acute exacerbation of the meningeal symptoms. Grace-Colvert (19) claims that the use of tuberculin in the subarachnoid space as a therapeutic measure in tuberculous meningitis may light up a solitary tubercle and give rise secondarily to a tuberculous meningitis. The acute symptoms described are undoubtedly due to the tuberculin acting on the allergic tissues of the leptomeninges. Lotti (20) reports a case of recurring tuberculous meningitis in a 22 year old patient who had attacks of greater or less severity since the age of 10 years. The occasional dissemination of tubercle bacilli and their products from a caseating tuberculoma of the brain into an allergic space would result in an acute exudative meningitis. Our experiments, taken with the fact that most if not all cases of tuberculous meningitis are secondary to some primary focus in another part of the body, assuredly indicate that a state of allergy exists in the subarachnoid spaces before the bacillus reaches the boundaries of the brain or its coverings.

Hypersensitive states of tissues are known to exist in infectious diseases other than tuberculosis. Studies pertaining to these phenomena in other diseases and their relation to certain central nervous system complications, particularly the complications following influenza, pneumonia, vaccination, etc., are in process of investigation.

#### SUMMARY

1. When living or dead tubercle bacilli and their products are placed in direct contact with the leptomeninges of hypersensitive (tuberculous) animals, there is a definite clinical and pathological response.
2. The clinical response is characterized by an onset of weakness, twitchings, convulsions and death of the animal within 6 to 12 hours.
3. Histologically the central nervous system shows an extensive



polymorphonuclear exudate distributed throughout the subarachnoid spaces of the brain and extending into the perivascular spaces.

4. The intensity of the response is directly proportional to the quantity of visceral tuberculosis or to the dose of tuberculin employed.

5. When small quantities of tuberculin are employed so as to permit the animal to survive longer than 24 hours, there is an exudate found in the sulci and at the base of the brain which is characterized by small lymphocytes.

6. The non-tuberculous animals when inoculated with tuberculin or tubercle bacilli revealed no clinical or pathological response. The tuberculous animals, on the other hand, when inoculated with glycerine broth always responded by a definite but slight polymorphonuclear exudate.

7. The possible relationship of the allergic state to postinfectious complications of the central nervous system is discussed.

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EXPLANATION OF PLATE 10

FIG. 1. Section of brain from a tuberculous guinea pig that received 0.3 cc. of 1/100 dilution of tuberculin. The Sylvian fissure is distended and densely compact with a cellular exudate.  $\times 45$ .

FIG. 2. Exudate at base of brain with perivascular and subpial infiltration.  $\times 70$ .

FIG. 3. Section of brain from a tuberculous guinea pig that received glycerine broth *via* basal cistern. Slight cellular response along borders of Sylvian fissure.  $\times 45$ .











## I. THE PERMEABILITY OF THE WALL OF THE LYMPHATIC CAPILLARY

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PLATES 11 TO 13

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Little is known of the permeability of the wall of the minute lymphatics. The neglect of this theme is the more remarkable in view of the great amount of work that has been done on the blood capillaries; but it is to be explained by the technical difficulties of experimentation. Recently in our laboratory a method has been devised whereby the most minute lymphatics can be disclosed and the permeability of their walls subjected to test.

In brief our method has been to render the lymphatics visible by means of well tolerated vital dyes, draining away in them from the point of injection, and to utilize these latter in the study of exchange between the lymph and tissue fluid. Under the circumstances of ordinary vital staining by a distribution of dye from the blood to the tissues the minute lymphatics remain invisible in the midst of the general coloration; but when a local injection of dye is made, the minutest lymphatics deriving from the region become filled with colored fluid and stand out brilliantly from their unstained surroundings. In a tissue accessible to study without disturbance it is possible to test the permeability of the lymphatic wall by the use of dyes of graded diffusibility, to observe the change produced by stimuli of various sorts, and to carry out other investigations. Such a tissue has been found in the ear of the mouse.

The present paper is devoted to orienting observations and to the permeability of the lymphatic wall under normal conditions.

### *Method*

The ear of the living mouse is almost ideal for direct observation of the smaller blood vessels and capillaries (1). The central cart-



ilaginous plate serves as a background against which the overlying blood vessels stand forth sharply under suitable experimental conditions. A small amount of isotonic dye solution or a suspension of finely particulate material is injected into the skin on the outer side of the edge of the ear. The lymphatics, rendered visible by their dye-stained content, are studied. The technique of lighting and inspection has in general been that employed for investigation of the ear capillaries (1).

Young adult white mice were used, which had been kept for a month or more on the same adequate diet. Preference was given to those of long headed type, that is to say, those having large ears so set as to be easily spread upon the platform used in the observations. Anesthesia was induced 1 hour before the experiment by injection beneath the skin of the abdomen of a 2 per cent aqueous solution of sodium luminal, 0.0125 cc. per gm. of body weight. An additional 0.02 cc. per 10 gm. of body weight injected intraperitoneally brought about more rapid narcosis. This dose of freshly dissolved drug produces an even anesthesia lasting 2 to 5 hours. Occasionally, it was necessary to place a broad collar of cardboard about the animal's neck (2) to prevent scratching of the ear before stupor ensued. Throughout the experiment the anesthetized mouse lay on its belly in a frame so moulded of plasteline and wire gauze that the head and body were supported while the ears rested lightly upon small white porcelaine plaques set upon daises which were in the same horizontal plane, to rule out gravity as a cause for any differences in them. The ears were thinly coated with neutral paraffin oil which served the double purpose of holding them against the porcelaine without distortion or pressure and of greatly increasing visibility. A beam of light from a Leitz-Wetzlar carbon arc, cooled by passage through one or more filters, 5 cm. thick, of Magnus' fluid, was directed onto the ear by an adjustable mirror. The observations were carried out with a Spencer binocular dissecting microscope.

When dye was to be introduced, the skin was punctured on the outer side of the ear near its edge with a steel dissecting needle which had been ground to an extremely fine point. Through the little wound, either a micro-pipette or a 30 gauge platinum iridium injecting needle was thrust 2 to 3 mm. toward the base of the ear as superficially as possible. Dye solution was then slowly introduced in small quantity, resulting in deeply colored areas 1 to 4 mm. in diameter. Even before the injection was finished the lymphatics deriving from such an area had taken up the dye and colored fluid was seen to stream away toward the base of the ear, the outlines of the containing vessels being sharply demarcated thereby. When it was desired to render single lymphatic radicles visible, a Chambers micro-dissection apparatus and quartz micro-pipettes were utilized for the injection of an extremely minute quantity of dye.

The dyes were in the main those already utilized in this laboratory for studies of

the permeability of blood capillaries. They ranged in diffusibility from the swiftly diffusible Neptune blue (comparable to patent blue V in this respect) to pontamine sky blue which passes not at all through ordinary collodion membranes and only very slowly through the capillary wall. All were injected in isotonic solution, sometimes in sodium chloride solution, again in Tyrode's solution, and yet again in mouse serum diluted thrice with Tyrode's solution—to approximate the composition of the lymph of an extremity (3–5). In many previous experiments on the blood vessels the innocuous character of the dyes has been evidenced (6).

### *Appearance of the Normal Lymphatics*

A general description of the minute structure of the ear has been provided in a previous communication from this laboratory (1); but nothing was said in it of the lymphatics. Those rendered visible by their content of dye-stained fluid appear as irregular channels of highly various size and shape. They form an abundant intercommunicating network. Fig. 1 shows those draining a single colored area resulting from an injection under minimum pressure. Even the smallest lymphatic vessels disclosed by the injection are  $15\mu$  in diameter, far larger that is to say than the blood capillaries. Most of them are many times larger. They extend to the very margin of the ear without evident pattern or relation to the blood vessels. The lymph flowing along them toward the base of the ear collects into a few trunks lying as a rule in the tissue between the fan veins of the ear. These trunks are, if anything, narrower than the vessels that they drain. There is none of the gradual increase in size occurring in the veins. At intervals along the course of the lymphatics one sees characteristic bulbous swellings and under high magnification valves can be made out. There are many fine cross-channels without valves or swellings; but few of these are to be seen ordinarily, since the lymph current does not bring the dye into them. They can readily be rendered visible, however, by injecting the large lymphatics under moderate pressure. These can be pierced after they have been rendered visible by a content of colored fluid. Variations in the depth at which dye is placed in the tissue at the edge of the ear have revealed a superficial and a deep plexus of lymphatics, intercommunicating and both possessing valves. The one is in the corium, the other above the fatty tissue immediately overlying the cartilage (1).

No matter where the dye solution is injected, whether at the edge or middle or near the base of the ear, it is always transported toward the head, peripheral flow being prevented. We have repeatedly pierced individual dye-containing lymph channels and have introduced into them dye of another color, with the micro-pipette directed toward the periphery, but seldom have we been successful in forcing colored fluid past more than one or two valves without rupture of the vessel. Also with fine micro-spatulas directed by the Chambers device we have "milked" dye-stained fluid peripherally but only on rupture of the valves. In the extreme ear margin, however, the lymph channels are not guarded by these latter. Here it is possible to obtain a localized retrograde injection from dye proximally placed, and to demonstrate the existence of lymphatic closed loops and culs-de-sac next to the very margin of the ear. Even here the channels are numerous and far larger than the regional blood vessels. At no time has any evidence been obtained of direct communication between the lymphatics and the tissue spaces. Always the lymphatics have appeared to be completely closed.

The study of fixed and sectioned specimens has corroborated these findings. The preparations were obtained by injecting the channels in the usual manner with a gelatin solution warmed to 43.0°C. and stained with pontamine sky blue, after which the whole ear was fixed in Zenker's fluid. Pressure was required to inject the mass.

### *Lymph Flow*

The first demonstration of the lymphatics by means of dyes was accidental and while suggesting the later method, it also indicated the presence of a considerable lymph flow in the ear. In the course of work on the gradient of capillary permeability (6) a dye had been injected intravenously that formed aggregates while in the blood stream and blocked some of the finer arteries of the ear. Almost at once the emboli began to dissolve and as this happened lymphatics draining away colored fluid became visible. Under the circumstances of the later experiments, there was an active flow along the minute lymphatics of the ear, but since this derived from injected regions it cannot be taken as indicative of normal flow; where two lymph channels joined, however, one could often observe that a stream of dye-

containing fluid was displaced, diluted, and swept away by another stream, itself unseen, deriving from tissue remote from the region of injection. This occurred with remarkable rapidity. It could not have been due to the transmission of pressure from the injected area since the tissue of the ear is yielding (7). In certain instances blue and red dye solutions were introduced about 1/2 cm. apart at the margin of the ear. The lymph channels draining the regions coursed to a common trunk, and, carrying as they did different colored fluids, one could see at the point or points of confluence a blue stream joining a red one, or *vice versa*, the two streams flowing unmixed for several millimeters. The findings were not so free from objection as those in which colorless lymph joined that which was colored, because both tributary regions were abnormal.

In twelve experiments a crystal of pontamine blue or a minute droplet of the dye solution was placed in the tissue at the edge of the ear with the aid of a Chambers device and a micro-spatula or injecting pipette. The dye dissolved to form small colored dots, and the margins of these gradually extended in the direction of the base of the ear, and in this direction only, as could be told by measurements with a micrometer scale. In addition to a drainage away of colored fluid by the lymphatic channels described, one saw small streamers of dye, not sharp-edged as with fluid confined in lymphatic channels, but with hazy margins, as if free in the tissue, extending in the direction of the base of the ear. As long as the dot of dye continued to be perceptible as such, its margin continued to advance in this direction, while at the same time the tissue on its outer side which had at first been included in it became colorless. A true migration of the dot had taken place. It gradually became lighter and smaller and within 18 hours after its formation, all traces of it were lost. It is plain from these observations that besides lymphatic drainage and an attendant rapid turn-over of the fluid within the tissue spaces, there is some interstitial flow toward the base of the ear.

The demonstration of the lymphatics by means of the dyes was practically always incomplete. A current does not course through all of the minute lymphatics at the same time, the fluid in some of them being at a standstill. The existence of extremely narrow cross-connections between large lymphatics which had appeared to have a

wall with no openings into it was repeatedly revealed by pressure manipulations. These connections allowed only the finest thread of colored fluid to pass through, and there is every reason to suppose that under ordinary circumstances they were closed off. That they had adequate walls was shown by their sharp outlines and by the fact that their contents even when under slight pressure did not pass into the surrounding tissue. They had no valves or bulbous swellings. The facts suggest that they were accessory lymphatics, not closed off temporarily as are certain of the blood capillaries (8), but channels unutilized under ordinary circumstances.

Continual observation over periods of 3 to 5 hours revealed no movement of the lymphatics; their outlines and relations to one another remained unchanged. The bulbous dilatations appeared rigid, as if their walls were moored in the position of distension. In the region between them, small puckerings, possibly caused by struts of connective tissue, seemed permanently to distort the vessel contour. When India ink or "Hydrokollag," dialyzed against Locke's solution, was injected into lymphatics, these cleared themselves within a few minutes but black particles were left adherent to their walls, outlining them sharply. In such preparations, which can be studied for a much longer period than lymphatics outlined with dye, no contraction of the wall has ever been seen. This is not because the contours of the wall cannot alter. In experiments on the effect of heat and cold, to be detailed in a subsequent paper, marked changes in the size of the channels have been observed. But it must be concluded that the lymphatics of the mouse ear unlike those of the wing of the bat (9) do not actively contract or relax under ordinary circumstances.

### *Permeability of the Lymphatic Wall*

Our first observations showed the walls of the lymphatics to be highly permeable. Despite the fact that fluid was continually entering them from the surrounding tissue, as shown by observations already mentioned, even the most indiffusible of the dyes we utilized passed out. A systematic study of the permeability thus disclosed was undertaken. For the purpose it was important to utilize ears which had not been injured or inflamed in the least. Those of nearly full grown animals of 16 to 20 gm. proved far the best, the ears of younger

mice tending to be too thick for useful observations and of older ones too often marred. Even in ears that appeared wholly normal, an ecchymotic dye escape, testifying to occult injury, was frequently met with. Fig. 2 illustrates this. Several sharply localized dye extravasations here and there along the lymphatics can be seen in the photograph. This irregular type of dye escape can be readily discriminated from that occurring everywhere along an uninjured lymphatic wall in the case of highly diffusible pigments.

The behavior of two groups of dyes was investigated, those of the one group (pontamine sky blue and Chicago blue 6B) being poorly diffusible, as judged by their behavior in the blood stream and by their failure to pass collodion membranes; those of the other (trypan red, brom phenol blue, and Neptune blue), more or less highly diffusible. Isotonic solutions of all these, in various diluents and at various strengths, were introduced into the lymph channels. Several aims were pursued. We sought to learn the effect of differences in the molecular concentration of the dyes upon the rate of passage from the lymphatics. Next we studied the influence upon dye escape through the lymphatic wall of variations in the protein and salt content of the fluid media carrying the dyes. And finally we ascertained whether known differences in diffusibility of the dyes as demonstrated by their passage out of the blood vessels and through gelatin *in vitro* (6) hold good as regards their escape from the lymphatics. A rough comparison of the relative permeabilities of the lymphatic walls and the walls of the blood capillaries, under the conditions of life, was thus carried out.

The molecular weights of the dye specimens, which had been purified of extraneous material, were calculated by the freezing point method with a Beckman apparatus. For the experiments fresh solutions isotonic with 0.9 per cent sodium chloride solution were made in twice distilled water. A 21.6 per cent watery solution of the pontamine sky blue we used was isotonic with blood, 17.1 per cent of Chicago blue 6B, 4 per cent of trypan red, 4 per cent of brom phenol blue, and 5.5 per cent of Neptune blue. Such isotonic solutions were mixed in varying proportions with the following vehicles:—sodium chloride solution 0.9 per cent, Tyrode's solution, mouse amniotic fluid, mouse serum, and a mixture of 3 parts Tyrode's solution with 1 part mouse serum—this fluid having the approximate protein content of lymph from a mammalian extremity, to judge from the literature. In a few instances rabbit serum concentrated fivefold was also employed. The material

injected contained varying percentages of dye, depending upon the purpose of the experiment, but as a rule from 1 to 2 per cent.

In previous experiments (1, 10), and in others to be reported in the following paper, these dyes have been used for vital staining of mice by intravenous injection, and have circulated in the blood in approximately the same concentration as was now used, that is to say 1 to 2 per cent. Variations in the percentage without change in the tonicity of the final mixture were accomplished by using isotonic diluents. Differences in the tinctorial strength of the dyes remained a factor which could not be controlled. Fortunately however, the differences in their diffusibility and those in the influence of the diluents upon their escape from the lymphatics far transcended the observed differences in tinctorial value so that these failed to complicate the interpretation of the results.

### *The General Phenomena of Dye Escape*

The phenomena which enabled us to recognize the passage of dye through the lymphatic wall were very striking. Ecchymotic dye escape has already been considered. The limitation of escape to certain segments of seemingly normal lymphatics sometimes occurs, its cause being as yet obscure. It is encountered only with poorly diffusible dyes. Ordinarily the passage of dye through the lymphatic wall takes place everywhere at approximately the same time and rate. Immediately after the channel has become filled with dye-stained fluid it appears sharply demarcated; but sooner or later, depending on the diffusibility of the dye employed, its outline grows misty. The coloring matter is escaping into the surrounding tissue, and in proportion as this happens the lymphatic becomes surrounded by a colored cloud and obscured.

Invariably in scores of experiments an increase in the concentration of the dye enhanced its escape from the lymphatic channels, irrespective of the character of the fluid in which it was introduced. In this respect the findings resembled those on intravenous injection. Comparison of Figs. 3 and 4 with Figs. 5 and 6 show how greatly a fourfold increase in the concentration of dyes increased diffusion.

### *Influence of the Fluid Vehicle upon the Escape of Dye*

Early in the work the fact was noted that the escape of the dyes was markedly conditioned by the character of the fluid in which they were dissolved. For this reason comparisons of the diffusibility of the dyes were made only when they were introduced in the same vehicle.

Some experiments specifically devised to determine the influence of the vehicle threw light also on the permeability of the lymphatic wall as compared with that of the blood vessels.

The several dyes selected for the work, pontamine sky blue, Chicago blue 6B, brom phenol blue, Neptune blue, trypan red, and vital red, were mixed in equimolecular amount with saline solution, Tyrode's solution, mouse amniotic fluid, mouse serum, a mixture of 1 part mouse serum and 3 parts of Tyrode's solution, and finally with rabbit serum, concentrated fivefold. The proportions used were 0.1 cc. or 0.2 cc. of isotonic watery dye solution to 2 cc. of diluent. Small amounts of two or three of these solutions were injected at different spots along the edge of each ear so that comparisons might be made in the one animal of the rate of dye escape as influenced by the various vehicles. At least fifty injections, and in some cases twice as many, were made with each dye in order to obtain ample data.

It was regularly found that the dye escaped most slowly through the wall of the lymphatics when it had been mixed with a fivefold rabbit serum concentrate. The material was prepared by ultrafiltration through collodion sacs (11). The other vehicles can be arranged in the following order, escape being progressively greater with each one: normal homologous serum, diluted homologous serum (1 part serum to 3 parts Tyrode's solution), homologous amniotic fluid, Tyrode's solution, and saline. The differences observed were very great. When pontamine blue was injected in a fivefold concentrate of rabbit serum, almost none got out through the walls of the lymphatics during the next 2 hours, though these vessels were deep blue with it; and in some instances no escape whatever took place in the hours during which the dye was draining away through them, that is to say before they became decolorized by drainage. A less considerable yet very marked retention resulted when normal mouse serum was used. On the other hand, when the dye had been mixed with amniotic fluid or serum-Tyrode solution mixture, definite escape through the lymphatic wall occurred in 15 minutes or less. In the experiment furnishing the photograph of Fig. 1, pontamine sky blue was injected in a serum-Tyrode solution mixture. With amniotic fluid and the serum-Tyrode solution mixture, dye escape took place at about the same rate.

When present in the lymphatics in Tyrode's solution without serum, pontamine sky blue passed into the surrounding tissue much more easily. Within 6 minutes a moderate perilymphatic staining was usually to be observed, such as that pictured in Fig. 3. Another 10 minutes saw a further spread of color.

By far the most rapid spread of pontamine sky blue occurred when 0.9 per cent saline solution was used as the diluent. Almost immediately after injection this dye, which is notably indiffusible, began to escape from the lymphatics.

The foregoing facts held true of all the dyes with which tests were made. The solutions employed constituted a series with decreasing protein concentration, and in proportion to this decrease the dye escape



increased. Only two protein-free vehicles were used, Tyrode's solution and saline. With the first of these the escape of dye was but little more considerable than when amniotic fluid or a serum-Tyrode solution mixture had been employed. When saline was used on the other hand, the dye escape was pronouncedly greater. It seems more than likely that the saline injured the lymphatic wall, rendering it unusually permeable. The fact is well known that tissues perfused with an unbalanced salt solution rapidly become edematous.

*Dye Escape from the Lymphatics Is Conditioned by the Diffusibility of the Dye*

When mixtures of the various dyes were brought into the lymphatic channels in equimolecular concentration and in the same fluid, considerable differences in the rate of their escape could be observed, and these accorded with the known differences in diffusibility of the substances *in vitro*. For example, Chicago blue 6B in Tyrode's solution passed through the lymphatic wall with greater difficulty than did an equivalent concentration of brom phenol blue, but with more ease than pontamine sky blue. The differences were found irrespective of whether the dyes were compared in amniotic fluid, serum, saline solution, or another vehicle.

The method of test was as follows:—Solutions of the pontamine blue, Chicago blue, brom phenol blue, and Neptune blue in a single fluid were made as already described, and injected. The moment of first escape of dye was noted with a stopwatch and the general progress and character of the diffusion studied. The behavior of three dyes was compared simultaneously in each of fifty animals by injecting all three into each ear. In fifty more animals only two injections were made into each ear, the behavior of two of the dyes being compared at one time. By shifting the injection sites, local influences were controlled. This precaution, though employed as routine, was unnecessary since the differences in rate and amount of dye escape from the lymph channels were so definite as to transcend the effect of local factors.

In other groups of fifty animals each, the behavior of dyes was compared when mixed with saline solution, mouse serum, and mouse amniotic fluid, and in serum diluted with 3 volumes of Tyrode's solution.

The concentrations by weight of isotonic solutions of pontamine sky blue and Chicago blue are nearly similar, 21.6 per cent and 17.1 per cent. Furthermore, the tinctorial qualities of the mixtures tested were not very dissimilar. As a result, reliable comparisons of the amounts of dye escape from the lymphatics could be

made under the microscope even after twentyfold dilution of the dyes by the various vehicles.

The relatively diffusible blue dyes, brom phenol blue and Neptune blue, were in watery isotonic solution, at about 4 per cent and 5.5 per cent respectively. When these solutions were diluted twenty times with the various vehicles, to yield mixtures comparable to those of the indiffusible dyes previously used, the dye-stained fluid within the lymphatics appeared distinctly weaker in color than pontamine sky blue and Chicago blue. But despite this fact, it was easy to detect the greater diffusibility of the coloring matter because escape from vessels containing it was so early and pronounced.

More effective comparisons of the rate of passage of the two groups of dyes from the lymphatics were obtained when the concentration of the dyes was increased, 0.2 cc. or 0.3 cc. of the isotonic dye solution being added to 2 cc. of diluent. The resulting solutions yielded decisive evidence of the differences in diffusibility already described.

The results of these experiments can be summarized briefly. Pontamine sky blue escaped from the lymphatics with more difficulty than any other of the blue dyes, and Chicago blue 6B was but slightly more diffusible. Brom phenol blue, trypan red, and Neptune blue passed out from the lymphatics with far greater ease, the last very swiftly.

The fact is well attested that the rate of vital staining with the acid dyes varies with their diffusibility as determined *in vitro* (12). So too does the rate of their passage outwards through the capillary wall (6). The present observations prove that the same holds true of their escape through the lymphatic wall. Furthermore dyes which escape from the blood capillaries with difficulty escape from the lymphatics with difficulty too, the ultimate limit of permeability being much the same for the two vascular membranes, despite the greatly differing hydrostatic and osmotic conditions under which they function.

#### DISCUSSION

Previous knowledge of the permeability of the minute lymphatics has been inferential in the main, being based upon comparisons of the blood and of the lymph obtained from ducts of sufficient size for cannulation. By our method the permeability of the wall of the smallest lymphatics can be directly tested. But the fact should be stressed that this permeability is tested in the direction opposite to that of normal flow,—a flow which, in our material, the ear of the mouse, is by no means negligible. It will be well, before discussing

the findings by our method, to sum up the conditions under which they were obtained. In the majority of our experiments the dyes were injected into the tissue of the ear in approximately the same concentration in which they circulate in the blood when injected intravenously for vital staining. In this concentration they have proved innocuous to the vascular endothelium. The lymphatics draining the injected area promptly filled with the colored fluid,—under negligible pressure in the circumstances of the case. It was the secondary escape of the dyes from these lymphatics that was studied. Their outlines as well as the character of the escape showed plainly that they are, physiologically speaking, well defined, closed channels, a fact which accords with the prevailing view of anatomists.

When a dye is injected into the ear of the mouse there is some gradual misty extension through the tissue from the region in which it is at first located, over and above the frank lymphatic drainage. This extension is in the direction of the base of the ear and is apparently interstitial in nature, the most careful observation failing to disclose that it takes place along definite channels. The lymphatics demonstrated by the dye are of very considerable size, in general far larger than the blood capillaries, and are as big at the margin of the ear as at the base, and, except for the trunks at the base of the ear, which do not let dyes through so readily, are equally permeable everywhere, irrespective of their size. There is, in other words, no gradient of lymphatic permeability.

It is plain that in general we have dealt, not with collecting lymph ducts but with the lymphatic capillaries or radicles. These take up dye so swiftly from an injected area that to all intents and purposes the latter may be thought of as giving directly into them, despite the evidence afforded by the experiments of an anatomical (and physiological) barrier. It would doubtless be possible with the finest micropipette to inject tissue without damaging the lymphatics; but under the circumstances of our work, and under those of injections through hypodermic needles one must suppose that some of the lymphatics are torn. That they do not close down like injured blood vessels but function at once for drainage is indicated by our findings. More will be said upon this theme in a later paper.

In view of the continuous and considerable passage of fluid into the

lymphatics of the ear in normal situations, as disclosed by the current in the channels, the escape of dyes in the opposite direction, that is to say from the lymph into the tissue, is especially worthy of remark. But the lymphatic plexus has a very large surface area, whence it follows that the fluid trend through the wall at any one point is negligible; and furthermore the wall is exceedingly permeable as our results show. These indicate further that salt solution is injurious to the lymphatic barrier and that the escape of dye through it from other fluids varies directly with the diffusibility of the dye, as manifested *in vitro*, and with the character of the fluid menstruum. The more blood protein the latter contains the less is the escape of dye, as would follow both from osmotic conditions and from the circumstance that dyes become in some part adsorbed upon blood proteins (13). The most indiffusible of the dyes we have employed (pontamine blue) is more diffusible than blood proteins, whence it follows that if it is retained by the lymphatic wall, as was the actual case, proteins will be retained also. That the escape of dyes from the blood stream is markedly conditioned by the amount of plasma proteins will be shown in a succeeding paper from this laboratory.

The fact is well recognized that substances of small molecule pass through the walls of the blood capillaries with such ease as to be to all intents and purposes unaffected in distribution by the direction of fluid flow (8). Any alteration of the blood salts and of sugar causes a practically instantaneous readjustment of their relations in plasma and tissue fluids. The most diffusible of the dyes that we have used is far less so than the substances just mentioned, and it is not surprising that while they appear very swiftly outside lymphatics carrying them, they do not immediately attain the same concentration outside as in. One might be disposed to invoke some special mechanism facilitating the passage of substances from the tissue spaces to the lymphatics, as against passage in the reverse direction, were it not that when the dyes are injected into the blood stream the same lag in their distribution is observed, although in this case under circumstances that would seem to favor escape. The fact that the lymphatics remain invisible in tissue stained by the distribution of dyes from the blood instead of being perceptible by reason of a more lightly stained contents is not surprising since poorly diffusible dyes spread so slowly in the interstitial

spaces that there is ample time for the lymph to color like the interstitial fluid, while to highly diffusible dyes the lymphatic wall is an inconsiderable barrier. Furthermore the general coloration would tend to obscure the lymphatics. All of the evidence that we have obtained supports the view that the permeability of the lymphatic wall resembles the permeability of the capillary wall in its essential features and perhaps in its degree.

Certain observations in the course of the work suggest that some of the existing lymphatic channels are not utilized ordinarily. The channels in question are very narrow and are unprovided with valves, yet are so sharply demarcated from the surrounding tissue that they must be considered as preformed channels.

#### SUMMARY

A technique has been developed for the demonstration of lymphatic capillaries in the ear of the mouse by means of vital dyes and for tests of their permeability under normal and pathological conditions. The lymphatics become visible as closed channels from which the dyes escape secondarily into the tissue. Some of them, cross-connections, with extremely narrow lumen, would seem ordinarily not to be utilized.

There is active flow along the lymphatics of the mouse ear under ordinary circumstances. The movement of dye was always toward the main collecting system. The valves of the lymphatics as well as fluid flow prevented distal spread. There was in addition slow migration, apparently interstitial in character, but in the same general direction, of dots of color produced by the local injection of dye.

The normal permeability of the lymphatics was studied with dyes of graded diffusibility. Their walls proved readily permeable for those highly diffusible pigments that the blood capillaries let through easily, but retained those that the latter retained. Finely particulate matter (India ink, "Hydrokollag"), they did not let pass. No gradient of permeability was observed to exist along them such as exists along the blood capillaries of certain organs.

The observed phenomena of lymphatic permeability, like those of the permeability of the blood capillaries, can be explained on the assumption that the lymphatic wall behaves like a semipermeable membrane.

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## EXPLANATION OF PLATES

## PLATE 11

FIG. 1. Ear of a living anesthetized mouse, photographed by reflected light, 16 minutes after the injection of 1 per cent pontamine blue solution in a mixture of mouse serum 1 part and Tyrode's solution 3 parts. The plexus of lymphatics, rendered sharply visible by their stained contents, lies in the corium. Other, deeper lymphatics can be dimly seen. No dye has passed out from the lymphatics though these have been full of heavily stained fluid since the injection was made 16 minutes before. The channels are wider than all save the largest of the blood vessels, which latter are visible in gray.  $\times 10$ .

FIG. 2. Ear of a living mouse photographed *in situ* 5½ minutes after filling the lymphatics with a 1 per cent pontamine sky blue solution in a mixture of Tyrode's solution 3 parts, and mouse serum 1 part. Several sharply localized ecchymoses of dye can be seen, though the channels are in general impermeable to it as yet. Immediately before photographing the ear a cover slip was placed over it.  $\times 8$ .

## PLATE 12

FIG. 3. Ear of a living, anesthetized mouse with the lymphatics containing 1 per cent pontamine sky blue in Tyrode's solution. 6 minutes after injection of the dye, its escape into the tissues from the lymphatics has just begun.  $\times 10$ .

FIG. 4. The same ear photographed 5 minutes later, that is to say 11 minutes after injection. The color has extended further from the lymphatics, owing to progressive escape of the dye and its secondary spread in the interstitial spaces.

Note that the dye escape is more rapid and diffuse than in the ear of Fig. 1. In

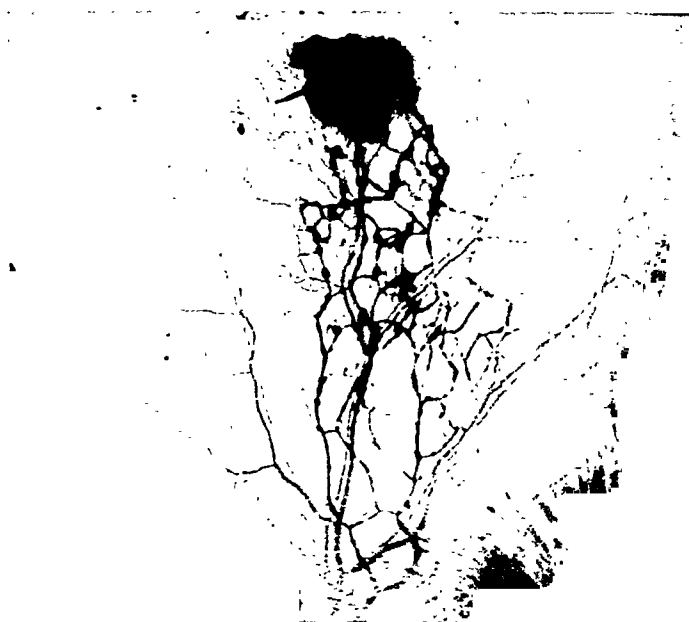
that experiment, a similar concentration of dye was injected but in a protein-containing vehicle.  $\times 10$ .

### PLATE 13

FIGS. 5 and 6. An increase in the concentration of dye within the lymphatics leads to its greater escape. The figures are to be compared with Figs. 3 and 4.

Ear of a living anesthetized mouse, *in situ*, injected with a 4 per cent pontamine blue in Tyrode's solution. The dye concentration was increased fourfold over that employed in the ear photographed in Figs. 3 and 4. The photographs were taken at the same time after injection, 6 minutes and 11 minutes respectively.

There has occurred a profuse escape of dye all along the lymph channels, with a marked secondary distribution through the tissue during the period between the two photographs.  $\times 8$ .



1



2

Photographed by Louis Schmidt

(Hudack and McMaster, I. Permeability of lymphatic capillaries)







Photographed by Louis Schmidt

(Hudack and McMaster: 1. Permeability of lymphatic capillary)





Photographed by Louis Schmidt

(Hudack and McMaster: I. Permeability of lymphatic capillary)





5



6

Photographed by Louis Schmidt

(Hudack and McMaster: I. Permeability of lymphatic capillary)



## II. INDUCED ALTERATIONS IN THE PERMEABILITY OF THE LYMPHATIC CAPILLARY

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PLATES 14 AND 15

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Lymphatic capillaries can be rendered visible by local injections of vital dyes. In the ear of the mouse, the channels, leading from an injected region, fill with dye and stand forth in deep color, coursing through unstained and undisturbed tissue. The method has been utilized for a study of the normal permeability of the wall of the lymphatic capillaries, as reported upon in an accompanying paper. The present study is concerned with the alterations in permeability that occur under various conditions, some of these within the limits of the normal, others frankly pathological.

### *General Procedure*

Mice, of 16 to 18 gm. body weight, under luminal anesthesia, were laid upon plasteline moulds and the ears were lightly spread upon white porcelaine plaques (see Figs. 1 to 3). Prior to experiment, the ears were carefully examined for gross or microscopic injuries and only those that appeared intact were utilized.

To render the lymphatics visible and to test their permeability as well, pontamine sky blue, a highly indiffusible vital dye, was injected into the tissue at the margin of the ear by a method already described (1). To approximate the probable protein concentration of the lymph, an aqueous solution of the purified dye, 21.6 per cent, which is isotonic with blood, was diluted to approximately 1 per cent with 20 volumes of a mixture consisting of 1 part mouse serum and 3 parts of Tyrode's solution. For brevity we shall call this standard pontamine solution. Under ordinary circumstances, the dye entering the lymphatics of the normal ear does not escape along their course until 12 to 15 minutes have elapsed, and then as a gradually increasing blue haze about the vessels. Changes in the rate of escape were taken to indicate alterations in the effective permeability of the lymphatic wall, though it is true that in some instances, to be commented upon further on, other factors may have been the conditioning ones. It was our practice to utilize one ear of each animal for experiment and the other as control.



Early in the work, the readiness with which the permeability can be altered prompted us to test whether the lighting system employed in the experiments had any such effect. As routine, the light from a Leitz-Wetzlar arc lamp, of 4 to 5 amperes, was filtered through 5 cm. of Magnus' solution (2) while still 60 cm. distant from the ear to be observed. The beam was then reflected to the object by an adjustable plane mirror.

The permeability of the lymphatics of ears exposed to well filtered light of high brilliance for 20 to 30 minutes was compared with that in others exposed to daylight only. No definite difference could be discerned. In the experiments the negligible influence of the filtered light from the arc was exerted upon experimental and control ears alike, since both were equally illuminated.

### *The Hydrostatic Pressure of Lymph*

Evidence of an active flow in the minute lymphatics of the mouse's ear (1) has already been given. It seemed well to investigate the pressures under which this flow takes place and the phenomena consequent upon obstruction. Accordingly, in a series of twelve experiments this was done.

An earlier paper (3) has described the utilization of small collodion bags connected with water manometers for the measurement of blood pressure in the smaller vessels of the mouse's ear. The same apparatus has been utilized for determinations of pressure within the lymphatics. The ear of the anesthetized animal was placed upon a horizontal porcelain plaque supported by a glass rod held by a Chambers device. A cylindrical collodion bag 3 cm. long and 2 mm. in diameter lay between the plaque and the ear in such manner that the outer third of the ear extended beyond the bag. The latter, slightly distended with water, was connected with a water manometer of 0.5 mm. bore, which in turn communicated with a reservoir and record syringe by which pressure changes were effected. Above the ear and the bag a transparent glass slide held by a rod in the Chambers device was adjusted parallel to the porcelain plaque below. With a fine camel's hair brush a neutral paraffin oil was then run between the ear and the upper platform and immediately the latter was brought down from above until it just touched the ear. With a little practice the adjustments were readily made and the zero point of pressure ascertained, while a second observer watched through the microscope. Slight increases in the distension of the bag led to a narrowing of the lumina of the lymphatics which had previously taken up the dye, and further distension occluded them. Simultaneous readings of the manometer told the pressure required to effect these changes.

Invariably in the twelve experiments the lymphatics were occluded when the bag pressure became equivalent to that exerted by a column of water 2 to 4 cm. in height.

*Mechanical Obstruction Increases Permeability*

The width of the blood vessels in the ear and the rate of flow therein were watched by one worker while the other raised the pressure just sufficiently to occlude the lymphatics. No narrowing of the veins coursing through the region pressed upon, or interference with the rate of flow through them, occurred as result of the slight pressure; yet when it was maintained there developed within the hour an edema of the ear, not marked but definite. The ear became slightly thicker, the natural folds of the skin appeared less deep and smoother, and with a blunt needle, "pitting on pressure" was demonstrable over the entire outer surface of the ear distal to the bag. Puncturing the skin with a fine sharp needle brought about the escape of a little free fluid. In six out of the twelve experiments standard pontamine solution was injected into the tissue at the edge of the ear after the barrier was placed. In these cases the lymphatics from the colored region filled with colored fluid, but none progressed beyond the barrier. Under the circumstances the lymphatics filled more slowly than usual and the colored lymph flowed off into a greater number of lateral channels than in unobstructed ears. In other experiments, the pressure barrier was placed on one ear after lymphatics of both ears already contained dye. In these instances the secondary escape of pigment through the lymphatic wall was perceptibly greater on the blocked side. The increased permeability was not accompanied by a recognizable dilatation of the obstructed lymphatics.

*Mechanical Stimulation Increases the Permeability of the Lymphatic Wall*

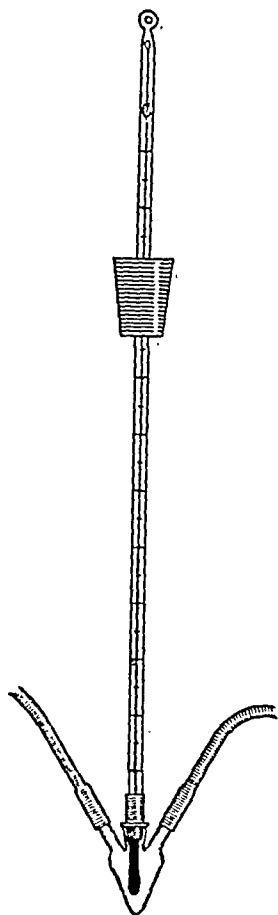
The permeability of normal lymph capillaries can be readily altered. When a light stroke is made with a blunt wire transversely across the surface of the skin on the outer surface of the ear of a mouse, there results an immediate and abundant escape of dye from the underlying lymphatics containing it. This escape is closely limited to the line of stroke (Fig. 1) and results in localized dye ecchymoses along the "tache."

To obtain uniform experimental conditions, the stroking was accomplished with a stout steel wire 5 cm. long and 1 mm. thick, affixed to a wooden handle and ground to a smooth, blunt tip. In all the experiments the strokes were applied by the

same worker holding the instrument at the same slanting angle and exerting as nearly as possible the same pressure. Mouse and plasteline form both rested in the scale pan of a spring balance. With a little practice a pressure during the stroke

of about 40 gm. as indicated by the balance was almost regularly exerted. Where the wire pressed upon the small blood vessels during the stroke, constriction occurred, leaving a barely perceptible white line. This rapidly disappeared and only occasionally during the next 2 to 4 hours could a slight narrowing, limited to the veins along the line of the stroke, be perceived with the microscope. Hyperemic flare occurred only with much heavier stroking.

At various intervals after the stroking, standard pontamine solution was injected in the usual way at the edge of the ear to render the lymphatics visible. In all the experiments each animal received but one injection of it.



TEXT-FIG. 1. Water chamber used for heating the ears of anesthetized mice. With the ear lying upon the glass bulb, water at any desired temperature was circulated through the apparatus. Even temperatures were maintained for varying periods with less than  $0.5^{\circ}\text{C}$ . variation.

Twenty-six of the animals were injected as soon as possible after the stroke or within 10 minutes thereafter. Practically at once after the lymphatics filled with colored fluid, that is to say within a minute after the injection, a profuse escape of dye began to take place from them where they crossed the line of stroke. Elsewhere the dye was retained. The photograph of Fig. 1 shows the lymphatic channels 6 minutes after the dye had begun to course along them, and 16 minutes after the stroking.

In another group of twelve animals considerable intervals were permitted to elapse between the stroking and the employment of the dye. Two of the mice were injected with the standard pontamine solution an hour after the stroking, and at half-hourly intervals during the next 5 hours two more were injected. The dye escaped from the lymphatics with the same ease an hour after the "tache" as immediately after it, and the first considerable lessening in escape was noted when the interval had

been  $2\frac{1}{2}$  hours. Intravenous injection of 0.05 cc. of isotonic 21.6 per cent pontamine solution, at approximately the same intervals, dis-closed, in the area of "tache," an increased permeability of the small blood vessels persisting for 5 hours and easily discernible after the lymphatic reaction had subsided. It is to be noted that the mice used in these experiments had a blood volume of approximately 1 cc. hence the dye circulating in the blood should attain a concentration similar to that of the dye in standard pontamine solution. A localized edema followed the stroke and invariably outlasted the changed state of permeability.

In further experiments of the same kind, dialyzed India ink and "Hydrokollag 300" were employed, either in place of pontamine blue, or combined with it. The graphite and ink particles rapidly adhere to the endothelial wall of the lymphatics of the normal ear, outlining the vessels clearly.

For the purpose Higgins' India ink was dialyzed through parchment paper and against Locke's solution for a week at  $2^{\circ}\text{C}$ . 15 cc. samples of the remaining ink were centrifuged—in 15 cc. centrifuge tubes—for  $1/2$  hour at approximately 3,000 revolutions per minute. The upper 8 cc. was then aspirated from each tube and portions added to Tyrode's solution for injection into the mouse ears. Usually 1 volume of ink to 3 volumes of Tyrode's solution sufficed to render the lymphatics plainly visible when filled with the mixture. "Hydrokollag 300" was prepared as described by Higgins and Murphy (4). Again, one to four mixtures of the particulate suspension and Tyrode's solution were used.

In twenty animals suspensions of India ink in Tyrode's solution were injected into the skin of the outer margin of the right ear 3 minutes after stroking the organ transversely across its middle. In the left ear, at a similar interval after stroking, suspensions of "Hydrokollag" were injected.

In ten animals both ears were stroked and an injection made into the right one, 3 minutes later, of India ink suspension in thrice its volume of standard pontamine solution. Into the left ears was injected "Hydrokollag" mixed with the same dye solution in the same amounts.

In no instances did the particulate matter escape from the lymph channels in the region of "tache," although the dye readily passed the endothelial barrier. A further attempt to ascertain the nature of the barrier was made by introducing mouse or rabbit hemoglobin as prepared by the method of Sellards and Minot (5), dissolved in Tyrode's solution. Six mice were utilized. In each the lymphatic

barrier let through hemoglobin in the region of "tache," within a minute after it appeared in them, whereas elsewhere they proved completely impermeable to it.

In a previous paper (1) the occurrence of dye ecchymoses from the lymphatics of apparently normal ears was described and pictured. They had the same general appearance as those elicited by stroking and it seemed probable that they might be due to essentially the same cause. To test the point six mice were given sodium luminal as usual, and before complete narcosis developed, the under surface of one ear of each was lightly tickled until the animal responded by scratching the upper surface, though not sufficiently to break the skin. A few minutes later when anesthesia was complete standard pontamine solution was injected into the skin at the margin of both ears. In every case on the scratched side, dye ecchymoses rapidly developed along the lymphatics, whereas none appeared on the control side.

### *Heat Increases the Permeability of the Lymphatics*

An extraordinary increase of lymphatic permeability resulted from the action of heat. It was applied in a variety of ways.

In 20 instances, one ear of the anesthetized mouse was dipped in water warmed to temperatures between 40° and 60°C. for periods varying from a few seconds to 5 minutes. Care was used to submerge only one ear and all other trauma was avoided. At varying intervals, from 2 minutes to 4 hours thereafter, standard pontamine solution was injected into the margin of both ears.

In another series of experiments, to observe the immediate effect of heat, the ear of the anesthetized mouse was placed upon the device shown diagrammatically in Text-fig. 1. A thin-walled glass bulb was moulded in a glass blower's flame to the shape of a flattened blunt spearhead. This bulb had three inlets, and one of its flat surfaces was painted with enamel. Two of the inlets served for the passage of water, while into the third a thermometer was inserted. Water, at any desired temperature, could be circulated through the apparatus while the ear of the mouse lay upon it under the binocular microscope. The white enamel gave the desired background and threw the structures of the ear into sharp relief. Thus, in 22 instances, the influence of temperatures ranging from 43–60°C. was tested on one ear, while the other ear lay upon a similar apparatus at room temperature.

In a few experiments one ear of the anesthetized mouse was subjected to a jet of warmed dry air, according to a procedure already described (6). Compressed air was blown above a gas flame into a funnel; and the ear was exposed to it as it emerged from the 3 mm. opening at the funnel end. A thermometer close to the ear gave approximate readings of the temperature.

The application of heat, even of so little as  $43^{\circ}\text{C}.$ , led to a rapid escape of the dye from lymphatics containing standard pontamine solution. A 5 minute exposure at  $43.0\text{--}43.5^{\circ}\text{C}.$  sufficed to cause escape in less than a minute after the lymphatics had filled with dye solution injected into the skin 4 minutes after heating the ear. In the unheated control ears no escape occurred for 12 to 15 minutes (1). Greater degrees of disturbance had proportional results. In these experiments, as in those involving mechanical injury, India ink and "Hydrokollag" taken up into the lymphatics failed to escape from them.

A typical experiment furnished the photograph in Fig. 2. Luminal was given to a mouse subcutaneously and after the customary interval of 1 hour the ears were placed upon the water chambers at room temperature. Water between  $43.0\text{--}43.5^{\circ}\text{C}.$  was circulated for 5 minutes through the apparatus supporting the right ear.  $1/2$  minute after the application of the heat, both arteries and veins showed dilatation which continued to increase while the ear lay upon the plaque. At the end of the period of heating, the water chambers were removed and 10 minutes later standard pontamine solution injected into both ears. At this time the left ear exhibited a mild contralateral reflex hyperemia. After another interval of 10 minutes the photograph shown in Fig. 2 was taken. Profuse escape of dye into the tissue had occurred in the heated ear, far more than on the normal side, and by this time, too, moderate edema of it had developed.

In all instances in which temperatures of  $43^{\circ}\text{C}.$  or more were applied, a vascular hyperemia developed almost at once, enduring for a variable period afterwards. The effect of this reaction on the gradient of permeability that exists along the blood capillaries has already been described (6). The reactive hyperemia developed during the same time as the increase in lymphatic permeability, as was found by the study of the animals in which the lymphatic channels had filled with dye solution prior to the heating. In all instances and with all the procedures used, a contralateral reflex hyperemia developed in the unheated control ear. The degree of reaction of the blood vessels on the two sides, though in general unequal, was often similar. The reactive hyperemia in the unheated ear was probably accompanied by a slight increase in the permeability of the lymphatics, judging from a comparison with the findings in mice which had not been subjected to heating.

*The Effects of Sunlight*

The pronounced changes in the permeability of the lymphatic wall elicited by mild stimuli led us to test the effect of still less considerable ones.

Nine mice were injected with luminal, and an hour later, at high noon, three of them were placed upon a gauze pad on a table standing near an open window in the full sunlight of a clear October day. A sunlit thermometer lying on the pad beside the animals registered 30°C. Three other animals were placed in a black cardboard box, also in the sun, and resting on a warming pad. These mice, though sheltered from the sun's rays, were surrounded by air at a temperature similar to that enveloping the animals directly exposed, that is to say between 29.6° and 30.5°C. Still another three animals, serving as controls, lay on a gauze pad in the middle of the room, out of the direct sunlight and at a temperature of 26°C.

After 15 minutes, standard pontamine solution was injected into the tissue at the margin of both ears of three of these mice, one of each group. The injections were made as rapidly as possible, beginning with the control mouse that had been in the middle of the room, and finishing with the animal exposed to sunlight. During the 3 minutes required for the injections and the subsequent 15 minute observation period, all three mice lay out of the sunlight and at room temperature.

The procedure was repeated at 15 minute intervals, the last mouse of the third batch of three having been exposed to sunlight for 45 minutes.

In all the experiments dye began to escape soonest from the lymphatics of the animals exposed to sunlight, although these were the last of each group to be injected and their lymphatics filled last with dye. In the animal exposed for 45 minutes, profuse escape of dye from the lymphatics occurred within 4 minutes, as compared with 17 minutes and 28 minutes for the control from the box and the one from the middle of the room. Its ears showed a mild hyperemia. That the increased permeability was due to the sunlight and not to temperature was shown by the relatively slow escape of dye in the case of the mice within the box. This was slightly more rapid than in the animals at room temperature, but did not nearly approximate that in the sunlight animals. A further experiment confirmed these findings.

Three further groups, each of three mice, were subjected to the conditions just described, but the ears of one group were injected and observed while the mice lay in the sunlight. Under these circumstances the dye escape from the lymphatics was even greater and more

rapid than before, in the experiments already described, occurring in 2 to 3 minutes. No escape took place from the lymphatics of the control animals for from 10 to 15 minutes.

### *The Effects of Chemical Irritation*

Xylol applied to the ear causes a profuse outpouring of dye from the lymphatics. The results of a few experiments will be briefly given.

The right ears of twenty-six anesthetized mice were painted once with Merck's chemically pure xylol applied by means of a camel's hair brush. From 5 minutes to  $\frac{1}{2}$  hour later standard pontamine solution was injected into the tissue at the margins of both ears. Within 1 to 2 minutes afterwards dye began to escape from the lymphatics of the painted ears and after an interval of 5 to 8 minutes the escape had become profuse. Meantime from the control ears little or no escape took place. Fig. 3 displays the difference in the ears of a mouse 23 minutes after a single application of xylol to the right ear and 8 minutes after injection of the dye.

Increased lymphatic permeability was noted irrespective of whether the dye injection was made after the painting with xylol or before. The increased permeability endured for a considerable time. For example, dye escaped within less than 2 minutes from lymphatics of an ear painted with xylol 4 hours previously. Repeated applications of xylol rendered the lymphatics still more permeable.

In 6 instances, about 15 minutes after painting both ears with xylol, India ink was injected into one ear and "Hydrokollag" into the other. The particulate matter taken up by the lymphatics remained within them—despite the increased permeability of the vessels for dye solutions.

The effect of xylol upon the blood vessels in the rabbit's ear (7) is well known. The vascular tree of the mouse's ear reacts in a similar manner, with an intense hyperemia. As in the experiments with heat, so in those with xylol, increased permeability of the lymphatics and reactive hyperemia develop approximately together. The phenomena are followed by edema of the ear. This latter, after a single application of xylol, is relatively slight, but after repeated paintings the ear becomes thick and the blood vessels appear as though seen through ground glass. When standard dye solution is injected subcutaneously, at once or within 2 hours after the last xylol painting, rapid and profuse escape from the lymph channels occurs, an escape far more pro-



nounced than that shown in Fig. 3. The ground glass appearance and thickness of the ear (2 to 6 mm.) prevent good photographic reproduction. The control ears, as in the heat experiments, show a contralateral reflex hyperemia.

*The Relation of Increased Lymphatic Permeability to the Development of Edema*

In all of the experiments involving the application of heat of 43°C. or over, an edema of the ear appeared. Several minutes elapsed between heating and the development of recognizable edema. During this period, both increased permeability of the lymphatics and an active hyperemia could be perceived. The reflex hyperemia developing in the control ears was frequently as intense as that on the experimental side, yet only occasionally did the control ear show any edema and this always of a very mild degree. The blood vessels of ears subjected to mild heating exhibit a great increase in permeability as we know from previous experiments, while on the control side vascular permeability is but little altered despite the induced hyperemia (6). This has also proved true on painting with xylol. Intravenous injections of 0.05 to 0.1 cc. of an aqueous 21.6 per cent, isotonic, solution of pontamine sky blue were made into a few mice after the study of the lymphatic permeability had been completed. The small blood vessels both of the control and the xylol-painted ears showed increased permeability, but in the latter far more than in the former.

*The Lymphatic Condition during Resorption of Edema*

What is the state of the lymphatic vessels during the resorption of edema? In the attempt to determine this, a procedure was sought which would give rise in the mouse's ear to a uniform edema lasting but a few hours. It was found that heating the ear for 5 or 6 minutes at 45-49°C. by means of the water chamber already described sufficed for the purpose. The organ was examined under the microscope at half-hourly intervals thereafter. Within 1 hour the ears showed a marked puffiness and swelling which had diminished at the 3rd and 4th hour and had quite disappeared by the next day, without signs of necrosis or vascular injury. The fluid had resorbed during this period. Some quantitative estimates were made of the amount and duration of the edema.

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Sixty young, adult mice weighing 16 to 18 gm. were anesthetized with luminal and the left ear of each was carefully amputated and weighed. Twenty individual ears taken previously from animals used for other purposes showed so little variation in the ratio of water content to total mass that the ears of these sixty were weighed at once as one group in a glass-stoppered weighing bottle. The right ears of all sixty animals were heated *in situ* at 46–49°C. for about 5 minutes. 2½ hours later half were amputated at the base and at once carefully weighed in one group. The heated ears of the remaining animals were amputated 5 hours after exposure and also weighed together. The ears were still edematous but not as thick or hyperemic as at the 2½ hour intervals after heating. The material provided by the three groups was desiccated to constant weight over phosphorus pentoxide at 56°C. The control ears showed a water content of 64.8 per cent. The heated ears amputated after 2½ hours, at which time there was a moderate edema, had a water content of 78.8 per cent, and those amputated after 5 hours, 76.8 per cent. The second figure given shows an increase in water content equivalent to 14 per cent of the total mass of the ear. In the following 2½ hours this had decreased by one-seventh. Subsequent observations have shown that the maximum edema occurs about 1 hour after heating. Amputation at this time would have revealed greater differences than those given.

Resorption had evidently begun within 5 hours after the heat stimulus. Lymphatic permeability at this period was now studied.

Five animals were anesthetized lightly with ether and one ear of each was heated on the water chamber at 44.5–45.0°C. for 6 minutes. Reactive hyperemia in the warmed ears and contralateral reflex vasodilatation in the control ears accompanied the procedure. The animals were allowed to recover from the ether. A moderate edema developed in the heated ears within the hour.

2½ hours later the mice were anesthetized with luminal and at varying intervals thereafter standard pontamine solution was injected into both ears. In three of the animals, edema was still present in the experimental ears 3½ to 4½ hours after heating, but none could be demonstrated on the control side. Dye failed to escape from the lymphatics of the heated ear in 30 minutes, indeed in one instance it was still retained after 50 minutes, while in the control ears it began to emerge in the usual period, 10 to 15 minutes. In the fourth animal, similar differences were found 3 hours after heating the ear. In the remaining animal, injected 4½ hours after heating, no edema was then present in either ear. Dye passed out of the lymphatics of both ears at the same rate as in normal animals.

In a further experiment one ear of each of five mice was heated at 49.5–50.0°C. for 1½ minutes with the animals under light ether anesthesia. 4 hours later the mice were given luminal, and when fully anesthetized, 4½ to 5 hours after the heating, dye was injected into both ears. To control the effect of contralateral reflex vasodilatation in the unheated (control) ears of the experimental animals, a group of normal mice were anesthetized and injected at the same time and in the same manner.

Similar results were obtained in all the experiments. At the time of dye injections, the heated ears still showed hyperemia and marked edema, the latter less than it had been 2 hours previously. The unheated ears showed slight hyperemia with a dubious or mild edema. In the ears of the control animals one could observe neither edema nor hyperemia. From the lymphatics of the latter, dye escaped in the usual time, whereas in the case of the heated ears of the experimental animals it failed to do so in twice or thrice the time. In these instances then, the most ready escape of dye was found in the mildly hyperemic, unheated ears of the experimental animals.

From these findings it can be said, during the resorption of the edema dye tends to remain within the lymphatics. It is conceivable that a brisk fluid movement into these vessels may account for the failure of dye to escape from them. In the edematous ears, during the period of fluid resorption, the lymph capillaries which at first filled with dye soon after became pale and faded from view. This phenomenon can best be interpreted as a washing out of them by fluid passing into them from without. It cannot be said that the experiments throw any light on the state of the lymphatics during fluid resorption but they indicate a participation of these vessels in the removal of fluid.

#### DISCUSSION

The lymphatics have been disregarded or overlooked in much of the current reasoning upon fluid transport and exchange. In part this is due to recognition of the fact that the lymph flow from not a few resting organs is negligible, and in other part to the unobtrusiveness of the lymphatics which, unless sought for, usually escape observation; but there is yet a third cause, namely that the means to demonstrate the minute lymphatics in the living creature and to study their physiology have not been available. In the present work the permeability of the lymphatic wall has been tested, though in the opposite direction to normal flow, it is true. Save when fluid is passing into the lymphatics in quantity (as during the resorption of edema), this should make little difference if,—as is ordinarily assumed for the wall of the blood capillary, and as the findings of our previous paper indicate,—the wall of the lymphatic behaves like a semipermeable membrane.

The results of our experiments leave no doubt that the barrier presented by the lymphatic wall, a barrier having under the conditions approximately the same permeability as the wall of the blood capillary, as shown by tests with vital dyes of graded diffusibility, is affected by many slight causes,—and more readily affected than the wall of the blood capillaries. Influences which come within the realm of the normal,—sunlight, slight warmth, a stroke, scratching which does not break the skin,—these greatly, if transiently, increase lymphatic permeability. It seems not merely probable, but certain, that such changes have a meaning for local conditions. Exchange between the blood and tissues is subject to alteration in a variety of ways—by vasodilatation or contraction, alterations in the systemic blood pressure, and so forth. The lymphatics in the nature of things constitute a more passive system; yet much of their usefulness under this or that condition must depend upon the state of permeability of their walls. It will be important to learn whether the active functioning of organs, with its attendant increase in lymph formation, affects this permeability.

None of the injuries that we have utilized to alter the permeability of the lymphatic wall breaks down the barrier so completely as to permit the escape of particulate matter; yet in so far as the lymphatic is rendered more permeable by this or that influence, it ceases to be a walled off channel. We have shown that slight stimuli render the lymphatic wall so very permeable that even hemoglobin passes it readily. What is true of hemoglobin will doubtless hold for proteins of smaller molecule, notably those of the blood plasma. Students of factitious urticaria and of wheal formation have been accustomed to explain whealing solely in terms of the escape of fluid from the blood vessels. But it is plain that if, as result of the injury causing the wheal, the lymphatics traversing the region implicated cease to be physiologically demarcated from the tissue because the barrier provided by their walls no longer exists, the fluid escaping from the lymphatics may readily contribute to the formation of the wheal. Evidence bearing upon this point will be provided in a subsequent communication.

The functional state of the lymphatics in tissues that are edematous for one or another of various reasons has great interest. Indeed,

until it is determined the mechanism of the edema cannot be wholly comprehended. We have shown that the edema which results from obstruction of the lymphatics is attended by an increased permeability of the wall of these vessels, and that during the formation of an inflammatory edema the wall of the lymphatics of the area involved becomes greatly more permeable. There are indications that the permeability returns to normal before the edema disappears, but for reasons that have already been given, they must be considered as inconclusive.

#### SUMMARY

A standardized solution of a vital dye which escapes with some difficulty from the lymphatics of the ear of the mouse has been utilized in tests of the permeability of the lymphatic wall under various conditions. It has been found that this permeability is subject to great change. The slight pressure that suffices to prevent lymph flow from the ear,—an organ in which such flow goes on normally,—soon results in increased permeability of the obstructed lymphatics without as yet any perceptible dilatation of these vessels. Mechanical stimulation as for example a stroke with a blunt wire, or scratching so light as not to break the epidermis, results in a practically immediate, great increase in lymphatic permeability, which is sharply localized to the region pressed upon. This increase in permeability, though so great that even hemoglobin is let pass by the lymphatics, endures but a few hours. Warming the ear to 43°C. or exposure to mild sunlight increases permeability considerably. Slight chemical irritation increases it greatly, though not so much that particulate matter is let pass. The edema developing as result of lymphatic obstruction or mechanical, thermal, or chemical stimulation is preceded by and associated with a large increase in lymphatic permeability.

The facts are discussed in relation to their bearing upon fluid accumulation within the tissue. It is plain that influences within the realm of the normal suffice to increase lymphatic permeability and that those which lead to edema cause a very great increase in it. In proportion as this increase occurs the lymphatics cease to be channels demarcated by a semipermeable membrane. It seems certain that the changes must be in some part responsible for the local accumula-

tion of fluid. There exist possibilities, on the other hand, of a correlation between the functionings of the blood and lymph vessels under certain pathological conditions, as during the resorption of edema.

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## EXPLANATION OF PLATES

## PLATE 14

FIG. 1. Ear of a living anesthetized mouse photographed by reflected light 6 minutes after the entry of standard pontamine solution into the lymphatic capillaries. 10 minutes previously the ear was stroked transversely across the middle with a blunt wire, as described in the text. Immediately before taking the photograph a fragment of cover slip was placed upon the ear, which had been coated with paraffin oil to increase visibility, as in the case of the other specimens photographed. Sharply localized ecchymoses of dye appeared along the line of "tache," although this latter was so weak as not to elicit any reaction of the blood vessels. Under normal conditions no such escape occurs in  $\frac{1}{2}$  hour.  $\times 8\frac{1}{2}$ .

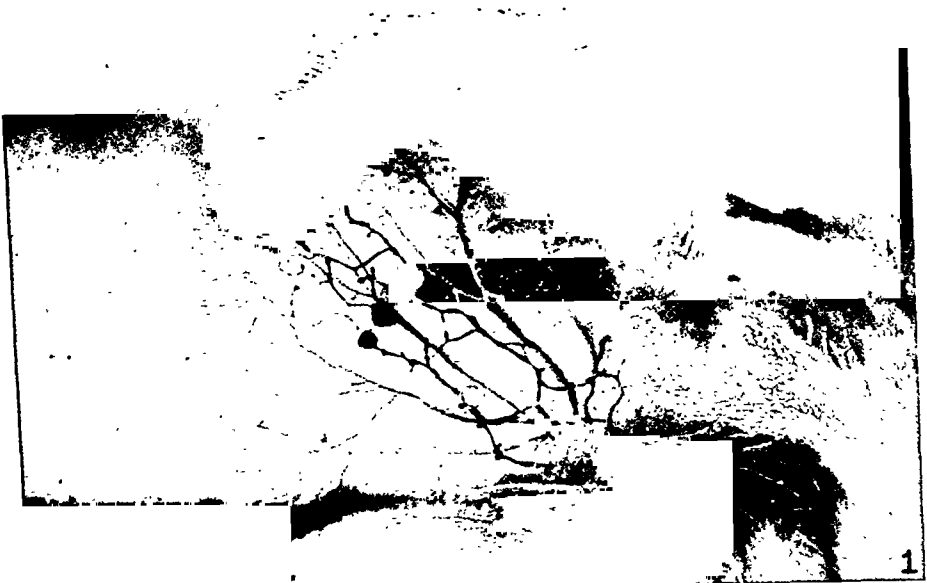
FIG. 2. The under surface of the right ear was warmed at 43.0–43.5°C. for 5 minutes, as it lay upon the water chamber described in the text. Both ears were then spread on plaques in the usual manner. 10 minutes after the heating, standard pontamine solution was introduced into the skin and taken up by the lymphatics. The photograph was taken after a further interval of 10 minutes. It will be seen that dye has escaped profusely all along the lymphatic channels of the heated (right) ear, while none has occurred in the control (left) ear.  $\times 3\frac{1}{2}$ .

## PLATE 15

FIG. 3. Ears of a living anesthetized mouse showing the escape of dye from the lymphatics 23 minutes after painting the right ear with xylol, and 8 minutes after the channels had taken up the standard pontamine solution. A marked escape of the dye has taken place from the lymphatics of the ear painted with xylol.

Both ears show a reactive hyperemia, that on the right the more severe. The lymphatics of the control (left) ear show slight dye escape the significance of which is discussed in the text.  $\times 8\frac{1}{2}$ .

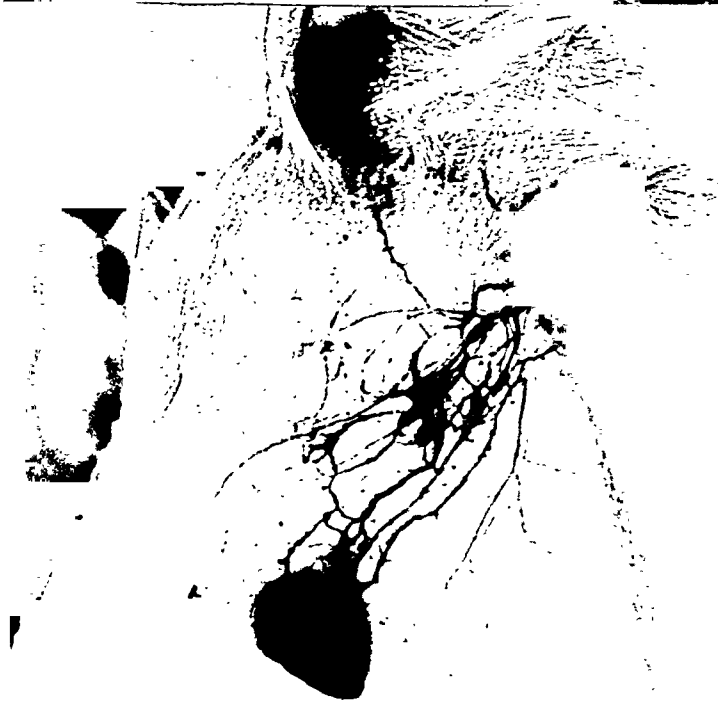




Photographed by Louis Schmidt







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# THE DEGREE OF COMPENSATORY RENAL HYPERTROPHY FOLLOWING UNILATERAL NEPHRECTOMY\*

## I. THE INFLUENCE OF AGE

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The gross enlargement of the remaining kidney which occurs after unilateral nephrectomy is called a compensatory hypertrophy.<sup>1</sup> Compensatory hypertrophy of the kidney is a relative term and designates the increase in size of a single kidney over that found when both kidneys are present. It is measured by the increase in the weight of the remaining kidney of an animal from which one kidney has been removed over and above the weight of one kidney of a control animal of the same age and sex maintained under the same conditions and subjected to an operation in which one kidney is exposed but not removed.

By the expression "degree" of compensatory hypertrophy we mean the maximum amount of enlargement which follows unilateral nephrectomy. Our work was done on the albino rat and in this animal the remaining kidney has reached its maximum degree of compensatory hypertrophy 40 days after the removal of the other kidney and the degree of compensatory hypertrophy thereafter remains constant. All the measurements in this paper were made 40 days after operation. But it is essential that some proof that compensatory hypertrophy is

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<sup>1</sup> This enlargement of the remaining kidney is due at least in part to a true hypertrophy of the parenchymal cells of the kidney, but in using the expression compensatory hypertrophy there is no intention to prejudice the question as to the possibility of a hyperplasia of the parenchymal as well as of the vascular and supporting tissue cells.

complete in 40 days be given now because until that point has been established our conclusions as to the relation between age and compensatory hypertrophy cannot be accepted. Without it the possibility would remain that the decrease in the degree of compensatory hypertrophy with advancing age might be only apparent and due to a slowing of the rate of increase in the weight of the remaining kidney in the older rats and not to any lack of ability to attain in the end to as great a degree of increase as is observed in the younger animals. And the necessity of dealing first with this point is the more stringent

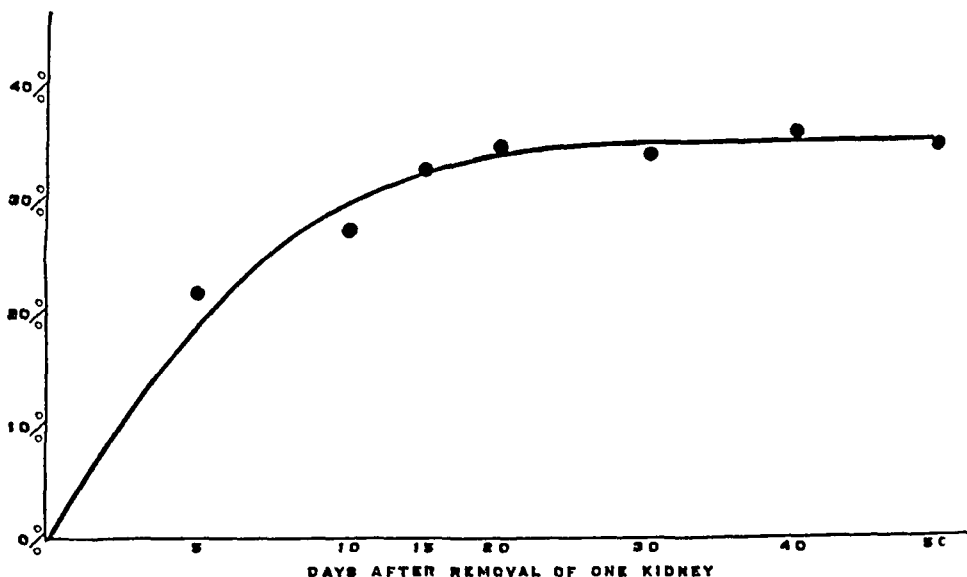


FIG. 1

since Smith and Moise (1) have been led to a different conclusion. They find that compensatory hypertrophy is a process which continues for at least 100 days after nephrectomy and their curve of the rate of compensatory hypertrophy never reaches any clear and definite maximum. However it happens that during the past 8 years we have collected for other purposes than those which concern this paper a large number of observations on the rate of compensatory hypertrophy of the kidney in rats of both sexes and of all ages. The result has been an adequate demonstration that when 40 days have elapsed after the removal of a kidney the remaining kidney has reached its

maximum degree of enlargement. Our results fall into two groups in accordance with differences in diet, in the ages selected for study, in the place where the experiments were carried out, and in the strain of albino rat which was used. We have selected from these two groups all observations in which all ages and both males and females were equally represented and in this way have constructed two graphs which summarize the findings on over 2000 rats. The ordinates represent the percentage increase in the weight of the remaining kidney over the average weight for one kidney of the controls. The difference

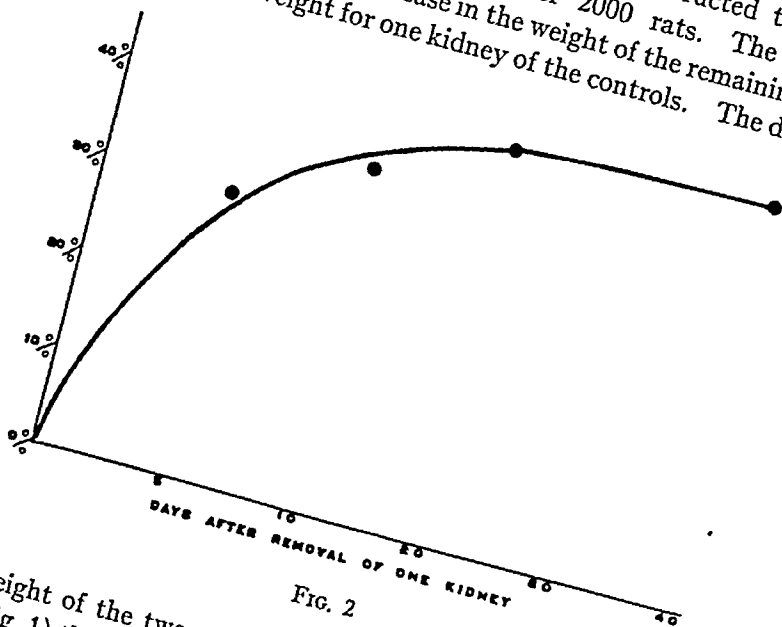


FIG. 2

in the height of the two curves arises from the fact that in the first group (Fig. 1) the study was extended to rats of greater age than in the second group (Fig. 2). It should be noted also that in other experiments, which could not be incorporated in these average curves because both sexes were not represented, observations have been continued to as long as 90 days after operation and that in these instances also the general result indicates that at all ages compensatory hypertrophy is complete in 40 days.

### Methods

The general methods used have already been described (2). Male albino rats were divided into control and experimental groups and one kidney was exposed or

TABLE I

Controls				Nephrectomized				Controls				Nephrectomized			
Body weight	Kidney weight	Kidney weight	Body surface	Body weight	Kidney weight	Kidney weight	Body surface	Body weight	Kidney weight	Kidney weight	Body surface	Body weight	Kidney weight	Kidney weight	Body surface
5 days old				15 days old				30 days old							
gm.	mg.	gm.	mg.	gm.	mg.	gm.	mg.	gm.	mg.	gm.	mg.	gm.	mg.	gm.	mg.
65	410	225	76	101	541	220	83	155	675	206	185	1066	289		
81	447	210	117	119	570	208	103	182	733	201	171	1044	288		
67	387	208	64	98	500	207	99	158	663	200	157	942	285		
105	503	200	111	125	589	207	122	159	653	196	178	991	282		
79	387	186	65	143	638	206	152	174	692	195	158	929	281		
75	372	186	89	146	644	205	124	173	676	192	177	1002	280		
82	393	184	111	115	518	193	123	183	695	190	160	912	273		
135	547	183	64	124	542	192	109	157	625	189	176	971	273		
78	373	181	77	152	621	192	120	171	657	188	142	834	271		
71	347	180	74	113	507	191	113	164	639	188	194	1031	271		
86	395	178	75	92	430	185	125	176	663	187	154	876	269		
84	388	178	76	124	514	182	117	169	642	186	160	895	268		
88	400	178	77	99	433	179	130	188	691	185	157	886	268		
70	338	177	68	85	392	178	114	160	616	184	165	912	267		
92	405	174		84	388	178	109	186	679	184	155	870	266		
70	331	173		141	541	177	109	165	624	183	132	772	262		
82	366	171		109	452	174	112	206	721	182	159	862	259		
92	392	169		132	504	171	119	186	670	181	159	858	258		
81	354	167		113	441	166	127	154	583	179	156	846	258		
77	332	163		105	413	164	119	170	614	176	159	847	254		
64	290	160					126	158	584	176	167	866	252		
89	351	155						158	572	173	191	946	251		
								159	567	170	183	913	249		
								171	583	167	166	845	247		
								166	569	166	130	709	243		

60 days old

220	872	211	165	965	282
209	836	210	199	1045	270
213	785	194	217	1079	263
188	717	193	218	1081	262
209	744	187	203	1018	260
259	860	186	223	1059	254
214	750	185	245	1126	253
220	761	184	213	1016	251
208	724	182	201	972	250
224	762	182	199	965	249
246	808	181	192	943	249
194	683	180	222	1032	248
227	752	179	203	973	248
200	694	179	191	929	246
215	729	179	198	945	245
225	748	179	176	871	244
286	882	179	182	865	237
241	782	178	186	875	237
221	735	177	199	917	237
209	697	175	215	967	237
221	715	172	182	848	232
276	805	167	172	799	228
252	749	166	195	861	225
222	670	161	157	734	222
			184	726	198

90 days old

267	997	212	289	1425	287
237	895	206	296	1337	265
233	877	204	252	1199	265
301	1037	203	266	1221	260
261	905	195	253	1177	260
259	894	194	298	1289	255
242	814	185	238	1080	248
316	977	185	238	1080	248
261	852	184	230	1058	248
298	922	182	261	1133	245
308	917	177	269	1157	245
288	874	177	253	1107	244
278	849	176	240	1055	241
290	875	176	281	1160	238
268	829	176	282	1157	237
231	748	175	290	1162	234
397	687	173	246	1033	232
285	850	173	259	1065	231
267	807	172	306	1179	228
262	797	172	256	1038	227
243	762	172	299	1129	229
273	805	169	258	1003	219
269	796	169	298	1085	214
208	649	163	257	878	192
285	795	162	212	771	191

180 days old

426	1238	193	351	1401	248
342	1041	187	318	1274	241
361	1050	182	334	1312	240
337	1001	182	389	1444	239
373	1052	179	361	1371	238
358	1025	179	321	1247	234
386	1079	179	327	1259	234
345	1001	179	320	1232	232
395	1067	175	318	1222	231
354	987	174	335	1266	231
352	987	174	401	1414	229
365	1005	173	345	1280	229
366	1000	172	332	1187	225
388	1038	172	332	1220	224
340	939	170	376	1318	223
416	1064	168	332	1211	222
310	858	165	347	1226	219
349	930	165	343	1214	218
341	940	164	346	1202	215
325	883	164	327	1141	212
333	872	160	321	1125	211
409	1001	160	322	1120	210
308	822	159	367	1215	209
392	905	149	326	1086	202



Controls				Nephrectomized			
Body weight	Kidney weight	Kidney weight Body surface		Body weight	Kidney weight	Kidney weight Body surface	
270 days old							
gm.	mg.	gm.	mg.	gm.	mg.	gm.	mg.
385	1143	190	379	1394	234		
382	1069	179	289	1158	233		
359	1027	179	345	1299	232		
282	873	179	384	1388	231		
357	982	172	343	1263	227		
377	1014	172	381	1348	226		
338	949	172	396	1385	226		
403	1050	170	327	1217	226		
441	1115	170	366	1288	222		
388	1019	169	346	1239	221		
422	1071	168	347	1236	220		
344	938	168	378	1304	220		
383	988	165	347	1226	219		
354	930	163	326	1180	219		
402	1009	163	346	1226	219		
334	874	160	359	1250	218		
433	1122	157	329	1178	218		
320	834	157	365	1260	217		
359	902	157	325	1153	215		
424	1006	157	418	1362	214		
402	966	156	471	1458	212		
336	854	156	300	1075	211		
487	1092	155	323	1123	210		
371	899	153	339	1128	204		
335	810	148	290	1008	203		
438	957	146	346	1070	191		
428	939	146	438	1244	190		
360 days old							
gm.	mg.	gm.	mg.	gm.	mg.	gm.	mg.
338	1139	207	309	1280	247		
339	1114	202	376	1431	242		
415	1255	199	318	1274	241		
367	1136	195	345	1332	238		
335	1061	194	399	1386	225		
312	1011	194	401	1385	225		
414	1216	193	419	1415	222		
303	944	184	377	1306	220		
364	1040	180	516	1594	218		
377	1051	177	352	1231	218		
429	1145	177	255	994	218		
439	1152	176	366	1255	216		
388	1055	175	339	1192	216		
347	969	173	374	1268	215		
354	981	172	284	1047	214		
354	970	170	282	1017	209		
307	859	166	365	1174	202		
401	995	161	406	1192	192		
353	913	161	359	1079	188		
343	882	158					
386	938	156					
406	925	149					
540 days old							
gm.	mg.	gm.	mg.	gm.	mg.	gm.	mg.
380	1233	207	376	1396	236		
452	1321	203	383	1387	231		
361	1092	190	309	1184	228		
316	982	186	413	1426	226		
472	1222	177	386	1362	226		
472	1192	173	381	1312	220		
536	1287	172	403	1328	215		
403	1067	172	282	1045	214		
392	1041	171	425	1364	212		
403	1059	171	460	1433	212		
426	1087	169	419	1344	211		
483	1176	168	378	1255	211		
400	1036	168	395	1272	208		
488	1183	168	496	1464	206		
418	1058	167	354	1158	204		
380	988	166	500	1444	202		
427	1054	164	348	1132	202		
511	1161	160	352	1135	201		
399	985	160	335	1094	200		
449	1057	159	380	1173	197		
511	1132	156	379	1163	196		
425	997	155	459	1314	194		
397	932	152	368	1104	189		

removed at 5, 15, 30, 50, 60, 90, 180, 270, 540, and 720 days of age. In the groups in which operations were performed at 5 and 15 days of age, it was necessary to clip the incisor teeth of the mother in order to prevent her from eating the young rats after the operation. All other groups received from the first the casein-starch-lard diet described as the experimental male diet (2). Each rat was anesthetized with ether 40 days after operation, exsanguinated, the kidney stripped of its capsule, and cut with a razor into two parts which were pressed between filter paper before weighing.

TABLE II

TABLE II																			
Age at operation		Age at death		Control group averages						Nephrectomy group averages						Mg. kidney per 100 sq. cm. body surface		Compensatory renal hypertrophy	
				No. rats	Initial body weight		Gross body weight at death		Corrected body weight	Body surface	Kidney weight	No. rats	Initial body weight		Gross body weight at death		Corrected body weight		Body surface
day	days	gm.	gm.		gm.	sq. cm.	gm.	gm.					gm.	sq. cm.	gm.	gm.			
5	45	22	—	89	82	214	387	14	—	84	79	206	617	181	299	118	65	2	
15	55	20	25	125	116	269	509	21	26	125	145	271	798	188	296	108	57	5	
30	70	25	42	179	170	347	644	26	42	173	164	340	903	185	266	81	43	7	
60	100	24	130	235	225	419	759	25	126	206	198	385	944	182	245	63	34	6	
90	130	25	166	272	265	468	851	25	169	272	265	467	1118	181	239	58	32	0	
180	220	24	290	366	361	575	990	24	281	346	341	554	1249	172	225	53	30	9	
270	310	27	322	384	381	596	976	27	324	361	356	569	1239	164	218	54	32	9	
360	400	22	318	372	367	581	1035	19	325	365	360	573	1255	178	219	41	23	1	
540	580	23	407	437	407	620	1100	23	389	398	390	606	1270	171	210	39	22	8	

## RESULTS

Table I gives the results. The kidney weight is expressed in milligrams per 100 sq. cm. of body size, since it has been shown that under uniform conditions this value is approximately constant at all ages (3). In calculating the body surface the formula of Carman and Mitchell (4) was used although more recent investigations (5) suggest that the constant should be smaller. However the magnitude of the constant has no effect on our relative figures. The kidney weights tabulated for the control groups are the average of the weights of both kidneys. The degree of compensatory hypertrophy is expressed as the percentage increase in weight of the single kidney over the average

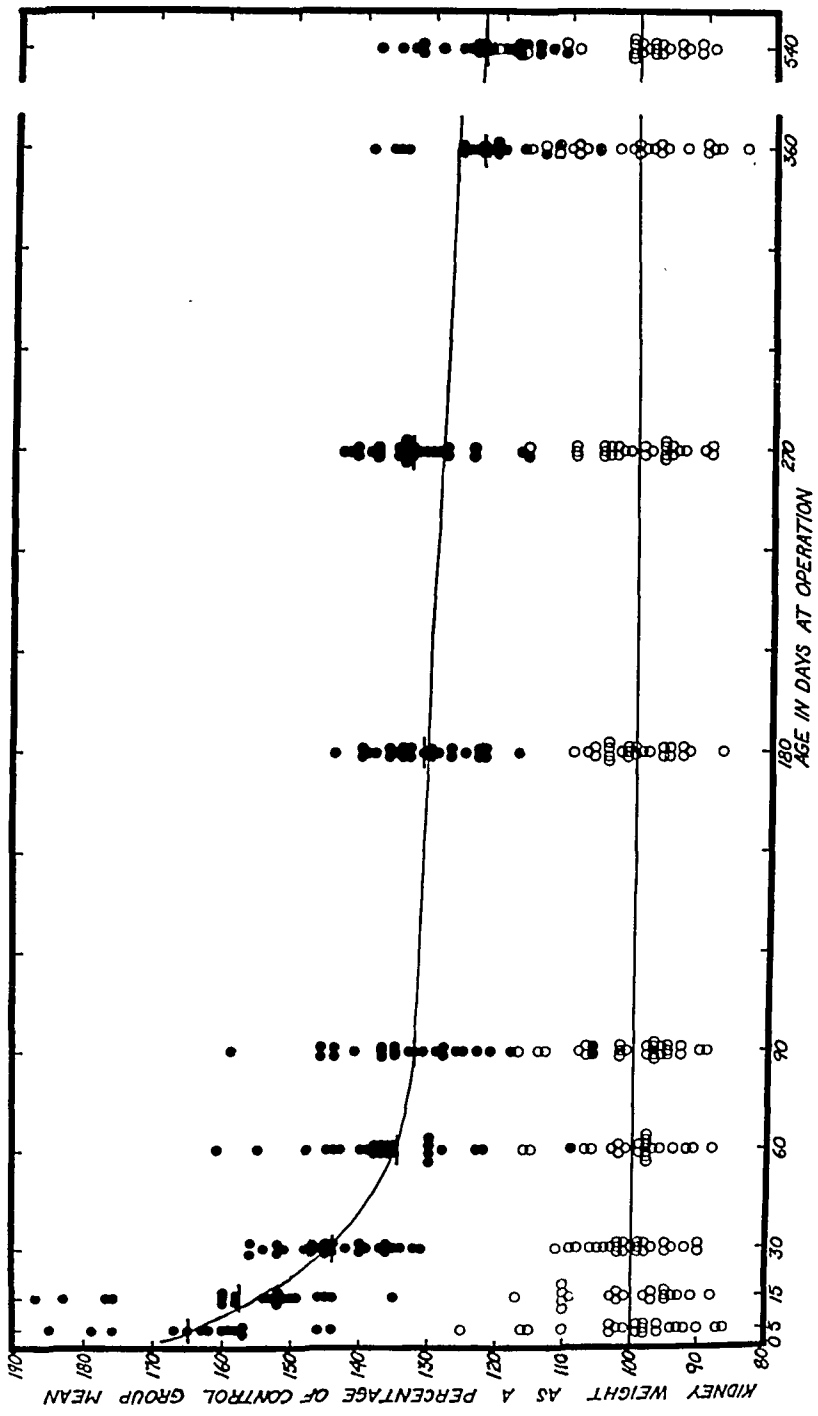


FIG. 3

weight of both kidneys of the control. In Table II the average results for each age are given. This table and the graphic representation of the individual observations given in Fig. 3, show how the degree of compensatory hypertrophy decreases, rapidly from 5 to 60 days of age, but thereafter very slowly as age advances.

## DISCUSSION

Young rats eat a greater amount of food in relation to their size than older rats. With a constant diet such as was given to all but

TABLE III

Age at operation	Control group					Nephrectomized group							
	Intake per sq. dm. per day		*Kidney weight per sq. dm.			Intake per sq. dm. per day		*Kidney weight per sq. dm.			Degree of compensatory renal hypertrophy		
	**Food	Protein	Calculated due to protein intake	As observed	Corrected for protein intake	**Food	Protein	Calculated due to protein intake	As observed	Corrected for protein intake	As observed	Corrected for protein intake	
days	gm.	gm.	mg.	mg.	mg.	gm.	gm.	mg.	mg.	mg.	per cent	per cent	
30	3.41	0.61	33	185	152	3.30	0.60	66	266	200	43.8	31.5	
60	2.41	0.43	23	182	159	2.43	0.44	46	245	199	34.6	26.9	
90	2.41	0.43	23	181	158	2.50	0.45	50	239	189	32.0	19.6	
180	2.07	0.37	20	172	152	2.11	0.38	42	225	183	30.8	20.4	
270	1.59	0.29	16	164	148	1.70	0.31	34	218	184	32.9	24.3	
360	2.11	0.38	21	178	157	2.16	0.39	42	219	177	23.0	12.7	
540	1.61	0.29	16	171	155	1.71	0.31	34	210	176	22.8	13.5	

\* One-half the weight of the two kidneys.

\*\* Average of last 10 days of experiment.

the first two groups young rats get proportionally more protein than the older rats. A definite relation has been shown to exist between the amount of protein eaten and the weight of kidney (6). Assuming that the protein-kidney weight formula is applicable to a single kidney it is possible from our food intake data to calculate what would have been the weight of the remaining kidney as well as of the average of the control kidney if the protein intake had been constant at all ages. The results of these calculations are given in Table III. They show that the essential features of the relationship are retained in spite of such corrections and that the decrease in compensatory hypertrophy

as age advances cannot be ascribed to the concomitant changes in protein consumption.

The concentration of water in body tissues decreases with age. Lowrey (7) has determined the water concentration in the kidneys of rats of varying age, and from his data a curve has been constructed which in Fig. 4 has been superimposed on a curve of compensatory hypertrophy derived from our data in Table I.

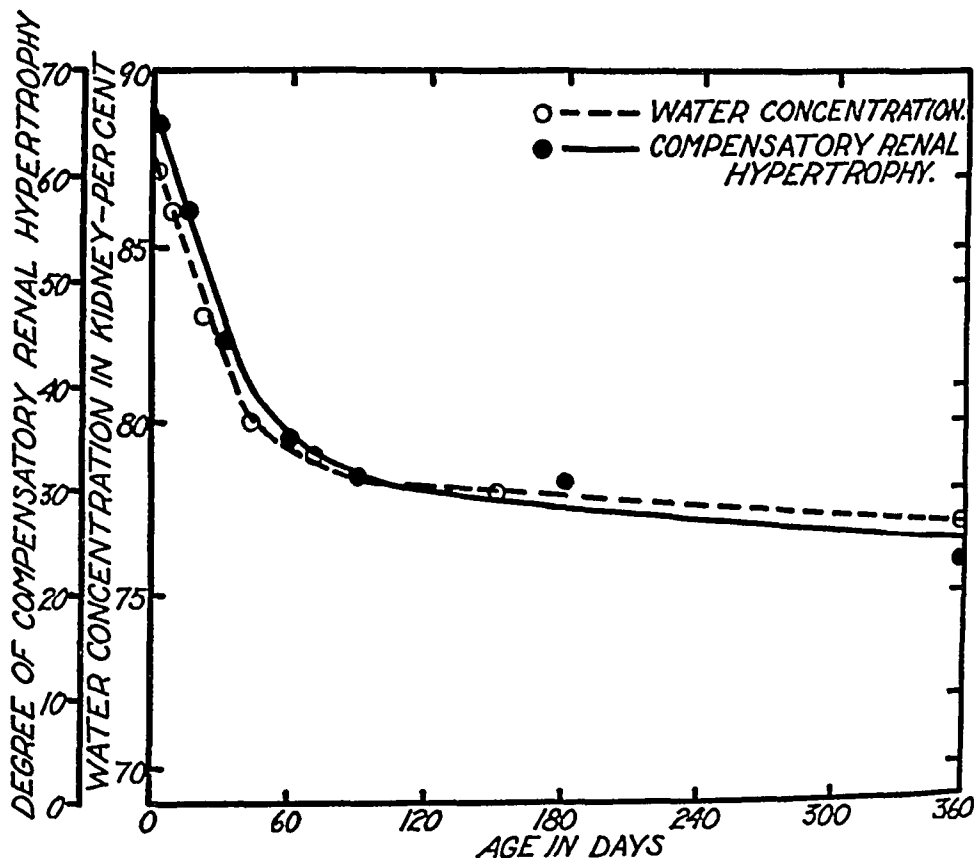


FIG. 4

It is interesting that there should be such a close correspondence between the degree of compensatory hypertrophy and the water concentration of the kidney at the time of nephrectomy. The agreement favors the view that the changes we observe are a special instance of the general decrease in growth capacity in the body as it grows old and that the design of any experiments made in the attempt

to elucidate the mechanism underlying these changes should be based on the general results of the study of senescence.

#### CONCLUSION

Compensatory hypertrophy of the kidney in albino rats becomes less as age advances. There is a rapid decrease from 5 days to 60 days of age and then a slow diminution throughout adult life.

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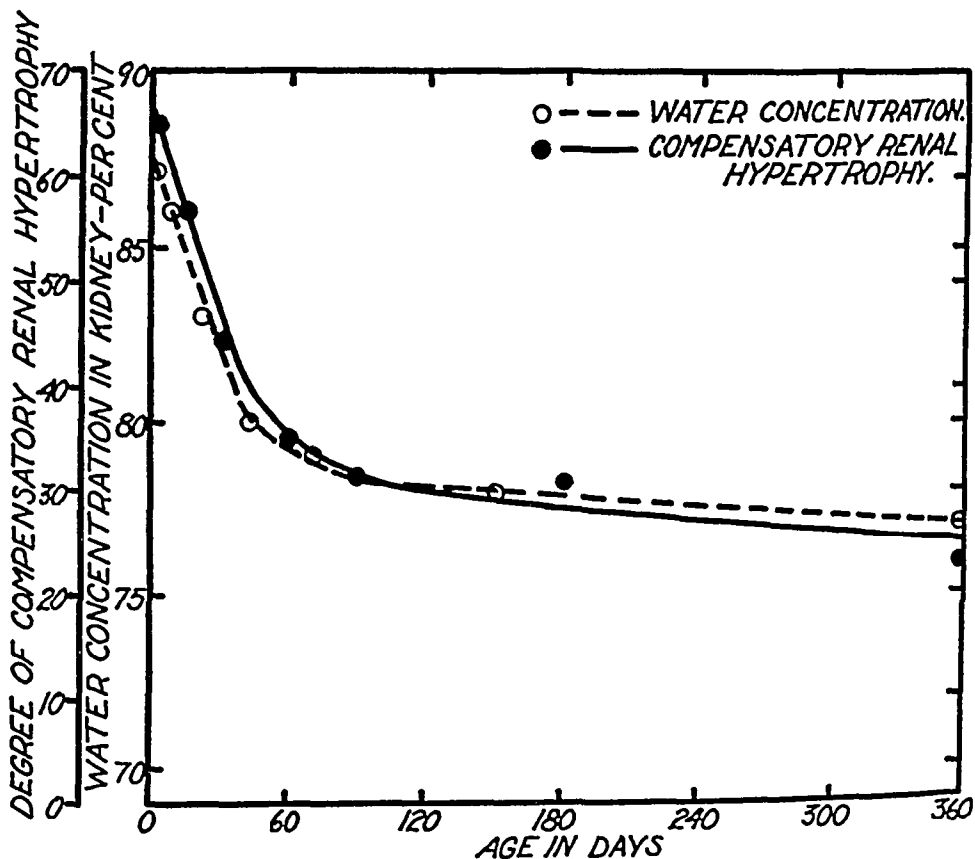


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THE PRODUCTION OF OSTEOGENIC SARCOMATA AND  
THE EFFECTS ON LYMPH NODES AND BONE MAR-  
ROW OF INTRAVENOUS INJECTIONS OF RA-  
DIUM CHLORIDE AND MESOTHORIUM  
IN RABBITS

By F. R. SABIN, M.D., C. A. DOAN, M.D., AND C. E. FORKNER, M.D.  
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PLATES 16 TO 18

(Received for publication, April 19, 1932)

Early in the use of the X-ray, it became evident that, on account of the repeated exposures, the operators were subject to grave danger. As is well known, they developed cancers from the burns in the skin and signs of damage to the blood-forming organs—anemia and leucopenia. These facts led both to experimental studies of the effects of X-rays and to an analysis of the changes in the blood cells (1-8).

In connection with these early studies on the effects of X-rays, it is of great interest to note the work of Murphy. Following the suggestion of Webb *et al.* (9, 10) that lymphocytes are a measure of resistance in tuberculosis, Murphy and his collaborators (11-26) made extensive studies on the relation of this strain of cells to resistance both to tuberculosis and to cancer. They first found that lymphocytes could be stimulated or depressed by means of X-rays, and determined the appropriate dosage. Nakahara (22) showed that the stimulating effect on the lymph nodes from small doses of X-rays began as early as 48 hours, as shown in his figures (Fig. 2, Plate 8, and Fig. 4, Plate 9). With these data in hand, Murphy and Morton (15) found that mice showing resistance to transplanted mouse cancer had a marked lymphocytosis and that this resistance could be broken down by doses of X-rays which produced no demonstrable changes except a necrosis of lymphoid tissues and the resulting lymphopenia.

When the clinical use of radium became common, it was found again that the danger was to those handling this material in account of the repeated exposures and that the effects were the same as with the X-rays. Mottram *et al.* (28) studied the blood of two groups of workers: those who prepared and measured the emanations and those who gave the radium to the patients. They found a rapid fall in both granulocytes and in lymphocytes and discovered that these





per cent transplants were successful and one tumor was in its tenth passage at the time of writing (1931).

Maisin and Dupuis (59) obtained sarcomata of enormous size in cocks and hens by intravenous injections of ionium, in combination with injection into the pectoral muscles of embryonic extracts. With injections of embryonic extract alone, or with arsenious acid, they could not produce tumors. One of the tumors metastasized; they did not succeed in transplanting them. Schürch and Uehlinger (60) implanted a needle containing 1 microgram of radium under the periosteum of the jaw-bone of a rabbit and allowed it to remain for 20 days. After 1½ years an osteogenic sarcoma developed, limited to the periosteum; it was made up of osteoblasts, and contained newly formed masses of bone and many multinuclear giant cells.

The literature considering the effects of radium on cancer cells in human cases will not be reviewed. It can be followed in the studies of Ewing (61), in the series of reviews published in the *Archives de l'Institut du Radium* (Vol. 1, Paris, 1927, to date), and in the files of the *Strahlentherapie* (Berlin, 1912, to date).

From the references here reported it is clear that radium has a destructive effect on the blood-forming organs; that the lymph nodes are more sensitive than the bone marrow; that there is a period of stimulation during which there is a relative increase in lymphocytes combined with a leucopenia; that the anemias tend to be of the primary type, associated with an increase in immature cells in the marrow; and lastly that radioactive substances produce malignant tumors, both sarcomata and carcinomata, some of which metastasize and can be transmitted in series.

In 1926 we began to study the effects of radioactive substances on the cells of the blood and connective tissues in rabbits. Through the courtesy of Dr. Frederick B. Flinn of Columbia University, radium chloride and mesothorium were obtained from the United States Radium Corporation. These compounds were put up in sealed ampoules of such a strength that 2 cc. of normal salt solution contained about 5 micrograms of the active material. In January, 1928, the strength of the material was tested for us by Dr. Alice H. Armstrong of The Rockefeller Institute and at that time an ampoule of radium chloride contained the equivalent of 5.1 micrograms of radium, and one ampoule of mesothorium, 7.7 micrograms. The dose of the mesothorium was also greater because it is known that it gives off 5 alpha particles to 4 from radium chloride.

losses were regained only after a considerable interval. There were then several reports of the study of the blood after exposure to radium (29-37) and recently it has become clear that the anemia due to radium may be of the primary type (27, 38-43).

The recent introduction of the use of luminous paint into industry, as well as the sale of radiated waters as tonics, has brought danger to another group of people from exposure to radioactive substances.

In 1929, Martland (44) gave the history of the industrial hazard involved in the painting of dials. Starting in Switzerland, the industry was transferred to this country, and in 1922, 1923, and 1924 there were deaths of dial painters unrecognized as due to radioactive materials. In 1924, Blum reported a case of necrosis of the jaw in one of the dial painters. There followed a series of studies of the people exposed to radium (45-52) in this industry. The early cases in this industrial group died of necrosis of the jaw; now, 8 years after the first reports, some of the cases have died with osteogenic sarcomata and some of those living have this condition, as determined by X-ray photographs. Flinn and his co-workers (49-52) have made special studies of the methods of detecting the presence of radioactive material in the living subject and measuring its amount. They have measured the rate of excretion and have tested methods of increasing this rate and determined the degree of safety of their application.

The development of osteogenic sarcomata in the human cases has increased the interest in the experimental production of tumors by radioactive substances. The first report of the production of osteogenic sarcomata in animals was published in 1910 by Marie *et al.* (53); they attempted to reproduce X-ray burns in animals to see if they would ultimately give rise to the same malignant reaction found in the X-ray operators. They exposed four rats to repeated small doses of X-rays for a period of several weeks. After 6 months one of them developed an ulcer which, 9 months later, suddenly became sarcomatous. The tumor showed active growth and invasion, but did not metastasize. In 1924, Bloch (54) reported the experimental production of epitheliomata of the ear in two rabbits by repeated exposure to X-rays. He succeeded in transplanting one of these tumors into another rabbit. The next year, Goebel and Gérard (55) reported the production of a neoplasm in one guinea pig out of twenty after repeated exposure to X-rays during 15 months. Daels and his coworkers (56-58) have made the most extensive studies of the experimental production of neoplasma with radioactive substances. They have produced sarcomata, some containing bone, and carcinomata of the bile ducts and the mammary glands. In all they have produced 10 sarcomata in 144 treated rats, 5 tumors in 80 treated mice, and 8 tumors in 191 treated guinea pigs. Of the 23 tumors, 19 followed the use of radioactive materials alone; the others developed after the use of a combination of radium and arsenious acid. One of the rat tumors was transplanted into 27 other rats; from 90 to 95

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## EXPERIMENTAL

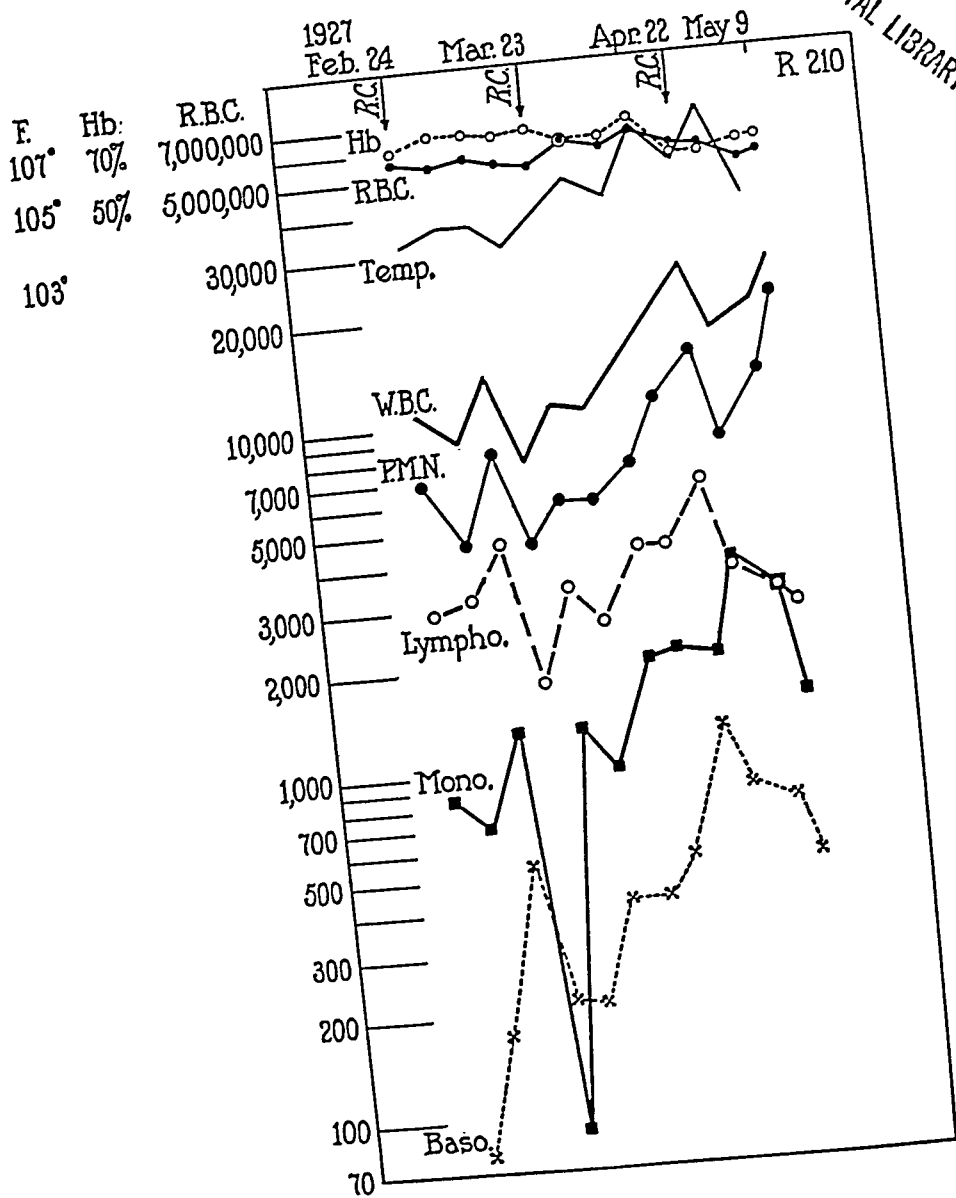
Nine rabbits were used for the experiment, five of which received radium chloride and four mesothorium. The injections were given intravenously once a month. The blood cells were counted once a week. The differential counts were made with the supravital technique, except during July and August, when they were made from fixed films. The changes in the lymphocytes are shown in Graph 2, in the red cells in Graph 3. The graphs are all on logarithmic paper. Only the seven animals that lived long enough to show these changes are included. All of the records opposite the numbered months are averages of the 4 counts. The average of the counts before any injection is shown on the upright line at the left. It represents from 5 to 15 counts covering from 1 to 4 months. For Rabbit R 211<sup>1</sup> there was only 1 preliminary count, 2,222 lymphocytes and 5,350,000 red cells, because this animal was substituted for one that died after one injection. From three to five injections were given in the spring, as shown on the graphs; then there was an interval of 3 or 4 months without injections which were resumed in September or October. All of the animals receiving the second series of injections, except R 211, which was killed in December, received an extra injection in January, 1928. One animal died 25 days after one injection; a second was killed after three injections (Graph 1); the other seven were killed at intervals varying from 11 to 19 months after the first injection. Each animal was killed when signs of some damage became evident, such as a fracture or marked weakness. The animals receiving the radium chloride survived the longest and showed fewer signs. The following are the protocols.

Rabbit R 134 was pregnant and 15 days after an injection of radium chloride gave birth to four young. It died 10 days later; the autopsy revealed edema of the lungs and extensive hemorrhages into the bone marrow and into the follicles of the spleen.

Rabbit R 210 received three injections of radium chloride. 6 days after the second injection, the temperature rose; 9 days later, the white blood cells had started to rise and in 2 weeks had reached 25,000 cells per cubic millimeter, without, however, any change in the percentages of any of the different strains (Graph 1).

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<sup>1</sup> These are serial numbers of the work of the department covering a term of years.



GRAPH 1



Then the neutrophilic leucocytes rose to 81 per cent and the lymphocytes fell to 11 per cent. There was no corresponding rise in the red cells nor change in hemoglobin. Except for the one count of 5,326, the lymphocytes were normal. These changes in the blood cells were due to an abscess on the back, on account of which the animal was killed 75 days after the first injection. It was in a good state of nutrition, considering the size of the abscess and the fever; it had gained in weight from 1,840 to 2,240 gm.

The bone marrow and the spleen reflected the presence of the abscess. The erythroid series in the marrow of femur, tibia, and humerus was represented by the usual masses of normoblasts. The fat had not been shifted out of the marrow, but the interstices between the fat cells were hyperplastic, due to an increase in neutrophilic B myelocytes (62). They were in active division and there was some increase in myeloblasts. In the sinuses of the spleen there was marked destruction of leucocytes within clasmotocytes and small masses of plasma cells in their neighborhood. There were also small groups of myelocytes, some of them in division. The lymphoid follicles of the spleen and the mesenteric lymph nodes were normal. The peripheral lymph nodes, on the other hand, were so markedly hyperplastic that almost no distinction could be made between follicles and sinus, as is clear in Fig. 1. The hyperplasia was due to the primitive cells rather than to any increase in mature lymphocytes. This accounts for the predominantly gray tone of the photograph, and is especially well shown in the lower follicle at the right which even lacks the rim of darkly staining small lymphocytes. The centers of all the follicles were filled with primitive cells, many of them in division (Fig. 2).

The protocols of the next five rabbits will be considered together. Three of them, R 157, R 171, and R 175, received the mesothorium, and two, R 133 and R 136, the radium chloride.

Three of these rabbits, R 136, R 171, and R 175, showed a marked shift in the lymph nodes from mature lymphocytes to a predominance of lymphoblasts. For example, in Fig. 5 is shown a small segment of a peripheral follicle from Rabbit R 171, in which the increase in the deeply staining lymphoblasts is clear. This reaction was even more marked in the lymph cords in certain nodes where all of the cells were small lymphoblasts. Wiseman (63) has shown that lymphoblasts may be small or intermediate or large cells. In all of these animals some of the nodes were markedly atrophied, as is shown in Fig. 3, Rabbit R 136. Figs. 1 and 3 are to be contrasted; they are from comparable nodes and are at the same magnification. In Fig. 1 is shown the phase of stimulation after three doses of radium chloride, and in Fig. 3 the aplasia following fourteen doses. A complete absence of lymph cords, such as is shown in Fig. 3, gave to many of these nodes a cystic appearance at autopsy. In the figure it will be noted also that there was a marked reduction in the peripheral follicles. The mesenteric nodes in some instances were represented by tiny nodules, some of them containing follicles but

others only lymphatic sinuses; these sinuses were identified by their endothelial cells which were loaded with the yellow pigment negative for iron, so characteristic a structure of the mesenteric nodes in rabbits. In one rabbit (R 171) some of the peripheral nodes could not be found. Fig. 4 illustrates a condition of great importance, namely, the marked damage of the stem cells; it is from a peripheral node of R 157. The darkly staining cells are not lymphoblasts, but normal lymphocytes which were printed dark in the photograph in order to show the pale damaged cells at all. In the supravital preparations from the nodes of this animal, both mesenteric and peripheral nodes showed a great reduction in all cells; those present were of two types, typical, normal, small lymphocytes and a second described as of the size of small lymphocytes but with an irregular cytoplasmic border and a nucleus in which no structure could be made out, except a dense nuclear membrane. In Fig. 4, these cells are readily identified as the so called pale cells of the follicles, or the stem cell of the lymphocyte. They are marked with arrows in Fig. 4 and are to be compared with the normal cell of the same type, also marked by an arrow, in Fig. 2.

The spleens in these animals showed for the most part a reduction in the lymphoid follicles, except in R 133, in which the follicles were in the state of hyperplasia illustrated for the lymph nodes in Fig. 1. In the sinuses there was a marked increase in iron-containing pigment.

The bone marrows were so mottled in appearance that the change is readily made out in sections with the unaided eye; this appearance is due to areas of aplasia alternating with zones which were either normal or in which there had been some shift toward the immature stages of the erythroid series. There was a change in the megalokaryocytes consisting in a large number of deeply basophilic nuclei almost completely denuded of cytoplasm; in one animal (R 157), 53 per cent of the megalokaryocytes were of this type. They may be considered as representing a shift toward immature stages of these cells. In almost every instance there was a marked change in the fat cells, consisting in a shrinkage due to the loss of fat from the cytoplasm. The bone marrow also showed phagocytic cells filled with pigment containing iron. There were certain special conditions not found in every animal, atrophy of the thymus, multiple abscesses, chronic bronchopneumonia, and certain changes in the liver. The changes in the liver consisted in hemorrhages, in a specific type of damage to the nuclei of the liver cells to be described in detail for Rabbit R 211, and in atrophy. Two of the rabbits showing marked damage of the liver had profound weakness of the muscles or paralyzes without signs of damage to the central nervous system.

Rabbit R 157 showed a complete consolidation of the upper lobes of both lungs. There had been a bronchopneumonia and large abscesses alternated with zones in which there had been new growth of bronchi and of the connective tissue septa, so extreme as to suggest a tumor. The cavities of the abscesses contained leucocytes and debris staining like lime salts. The zone showing the most marked changes in bronchial epithelium is illustrated in Fig. 10. Only a small row of typical bronchial epithelial cells (Arrow A) identifies this section as lung. Oppo-

site Arrow B is a solid mass of bronchial epithelium, suggestive of a tumor, but we interpret the condition as due to chronic inflammation.

All of the animals showed a loss in weight ranging from 300 to 680 gm. during the period of the final fall in lymphocytes.

Rabbit R 117 was one of the two animals which developed an osteogenic sarcoma. The animal had a litter of five young after the third injection and another litter after the sixth. During the period of the lymphopenia (Graph 2), the basophils fell and there was a loss of 680 gm. in weight. All of the bones, including the skull, were radioactive. The bones were tested for us by Dr. Armstrong, both in the fresh state and after being dried and ground.

The lymph nodes were all smaller than usual and looked cystic. The liver was small and the thymus markedly atrophic. The spleen was slightly larger than normal and weighed 1.6 gm. The most interesting changes were in the bones. All of them were extremely brittle. In the marrow of the left humerus there was a small white plaque near the epiphysis which proved to be a sarcoma (Fig. 7). There were many vessels between bone and marrow. The right humerus showed more profound changes; this marrow was more markedly adherent to the bone, but was cut out and represented a calcified cast of the marrow cavity. It showed no blood formation, no supravital preparations could be made, and the mass was put aside for decalcification. The marrows of the radii and ulnae were gelatinous and showed more blood formation than is usual in these bones. The femoral and tibial marrows were likewise gelatinous; the distal end of the tibia being most markedly so and as usual almost aplastic. The active marrow in these bones was deep red and showed the same increase in blood vessels between marrow and bone as in the humeri. Counts of marrow cells of the active areas were normal in the proportion of erythroid to myeloid cells (64, 65), and there had been no shift to immature forms in either myeloid or erythroid series.

Figs. 7 to 9 show the tumor of the humeral bone marrow. In Fig. 7 is the nodule and its border at a magnification of 120 diameters. The tumor, as shown in the left half of the photograph, was relatively uniform, made up predominantly of the sarcoma cells, but there were remnants of the sinusoids marked by perivascular clasmocytes filled with iron-containing pigment; there were also a few myelocytes, some of them in division. The nuclei of tumor cells varied in size but not in type (Fig. 8); their chromatin was in fine particles along the linin framework; they had conspicuous basophilic nucleoli. Many of the nuclei were in division, as is shown in Fig. 8. The border of the tumor shown on the right side of Fig. 7, and at higher magnification in Fig. 9 showed the invasion of the tumor cells into the marrow. In this zone the marrow was hyperplastic due to a marked increase in neutrophilic leucocytes. This is clear in Fig. 9, where the characteristic tumor cells marked by arrows are shown between the marrow cells which are predominantly leucocytes.

There was also a tumor in the left axilla, about  $2\frac{1}{2} \times 1 \times 1$  cm., which was made up of cells like those of the sarcoma of the marrow. In supravital preparations the cytoplasm showed no reaction to neutral red, as is characteristic of tumor cells.

The nucleoli were conspicuous. There were considerable numbers of multinucleated giant cells with nuclei of the same type. At one end there was a small necrotic area. There were three similar nodules in the lungs, two small ones in the left and a larger one in the right lung. Unfortunately these tissues were lost, but the supravital studies of the cells indicate quite clearly that there were metastases of the sarcoma. The study of the tissues of the next animal leads to the suggestion that the calcified marrow of the right side was an older stage of the tumor.

Rabbit R 211 was the other animal that developed a sarcoma. The sections are shown on Figs. 11 to 14. This rabbit received six intravenous injections of mesothorium during a period of 11 months, as is shown in Graph 2. In December the animal had a spontaneous fracture of the lower end of the right femur and was killed. Thus the experiment was the shortest of the series of seven which received the second series of injections. No anemia developed; the red cells which had averaged 5,400,000 for the 1st month were 6 million the last month. The final fall in the white cells during November and December was due mainly to lymphocytes; in small part to basophils. During this period the loss in weight was 300 gm. Almost the entire marrow cavity above the fracture was occupied by a tumor. The sarcoma replaced almost the entire marrow of the right femur. The contours of the tumor were sharp, as is shown in Fig. 11. At the upper end of the tumor there was a zone of aplasia (Fig. 11), beyond which there was a remnant of normal bone marrow. The fracture of the bone was opposite the lower end of the tumor. The sarcomatous nature of the new growth is clear in Fig. 11. The type of cell is shown in Fig. 12; the nuclei are of the same type as in the tumor of R 117. In many places there were small masses of newly formed bone in the tumor, some of them in small plaques, such as the one shown opposite Arrow A in Fig. 12, others in irregular networks. Near these small masses of bone were giant cells, such as the one marked by Arrow B, Fig. 12, which is of the type of the osteoclast. The nuclei in these giant cells were like those of the tumor cells, which is in agreement with the theory of Arey (66) that the osteoclast is formed by the fusion of osteoblasts.

That the tumor itself was derived from osteoblasts is indicated in Fig. 13, which is a small mass of bone against the tumor; it is denuded of osteoblasts, but three typical tumor cells nearby probably represent them. Fig. 14 is a small piece of damaged bone near the fracture. It was cut without decalcification; the bone corpuscles are shown with their processes entirely free from the canaliculi of the bone and in the upper part of the section the mineral matter of the bone is shown much fragmented.

The bone marrow in this animal does not appear mottled with the development of the aplastic zones seen in other animals. The cells, however, are difficult to discriminate because of a damage to the nuclei which makes them all look alike. Mottram (67) has seen the same change in the bone marrow of rats exposed to the gamma radiation from radium. The fat cells and the matrix were normal. A count of marrow cells with the supravital technique showed an increase in the

proportion of erythroid elements, 1,023 erythroid to 990 myeloid. Of the erythroid cells, there was an increased proportion of the early erythroblasts (18 per cent), with late erythroblasts 29 per cent and normoblasts 52 per cent. The early erythroblasts were described as very large cells which were entirely typical. 31 per cent of the megalokaryocytes were of the type already described, represented by deeply staining nuclei, apparently denuded of cytoplasm. Some of the less darkly stained nuclei showed marked signs of damage to the chromatin.

All of the lymphoid tissues including the follicles of the spleen, the mesenteric, and the peripheral nodes were small but in sections were hyperplastic, owing to the same marked stimulation of the primitive cells seen in Rabbit R 210. None of these cells, however, were in division, and both in the spleen and in the lymph nodes there were large masses of them, showing a peculiar type of necrosis. This process involved only the pale stem cells. The reaction is illustrated in Fig. 6; in the photograph the nuclei are so pale that they are difficult to differentiate and in the section stained with hematoxylin and eosin the entire area had a slate blue color. The cells look as if there had been a complete solution and diffusion into the tissues of the stainable matter of the nuclei. This is the only animal in which such extensive necrosis of lymphoid tissues was found. In the mesenteric nodes there were large areas covering several low power fields showing this necrosis; some of the small peripheral nodes were completely involved and the entire thymus showed the reaction. Besides this reaction in the lymphoid tissues, there was a similar extensive damage to the liver cells. Most of the cells of this organ showed the same slate blue color of the cytoplasm with signs of damage to the chromatin of the nuclei; but the nuclei were much less depleted of chromatin than those of the primitive cells of the lymphoid tissues.

#### DISCUSSION

In this series of experiments a number of phenomena have been observed: marked changes in the lymphoid tissues eventually registered by a lymphopenia; a slight anemia associated with a destruction of red blood cells and, within the limits of this experiment, with only minimal changes in the bone marrow; atrophy of the thymus; a damage to the liver cells and a reduction in size of this organ; the frequent development of abscesses; one spontaneous fracture; and in two instances the development of osteogenic sarcomata. Throughout this discussion it is to be borne in mind that the number of animals used was small.

All studies on the biological effects of radioactive substances show that the dose is an important factor; as shown in the literature, depending on the dose, the effects have ranged from conditions in which only lymphoid tissues have been altered (44) to death in a short time

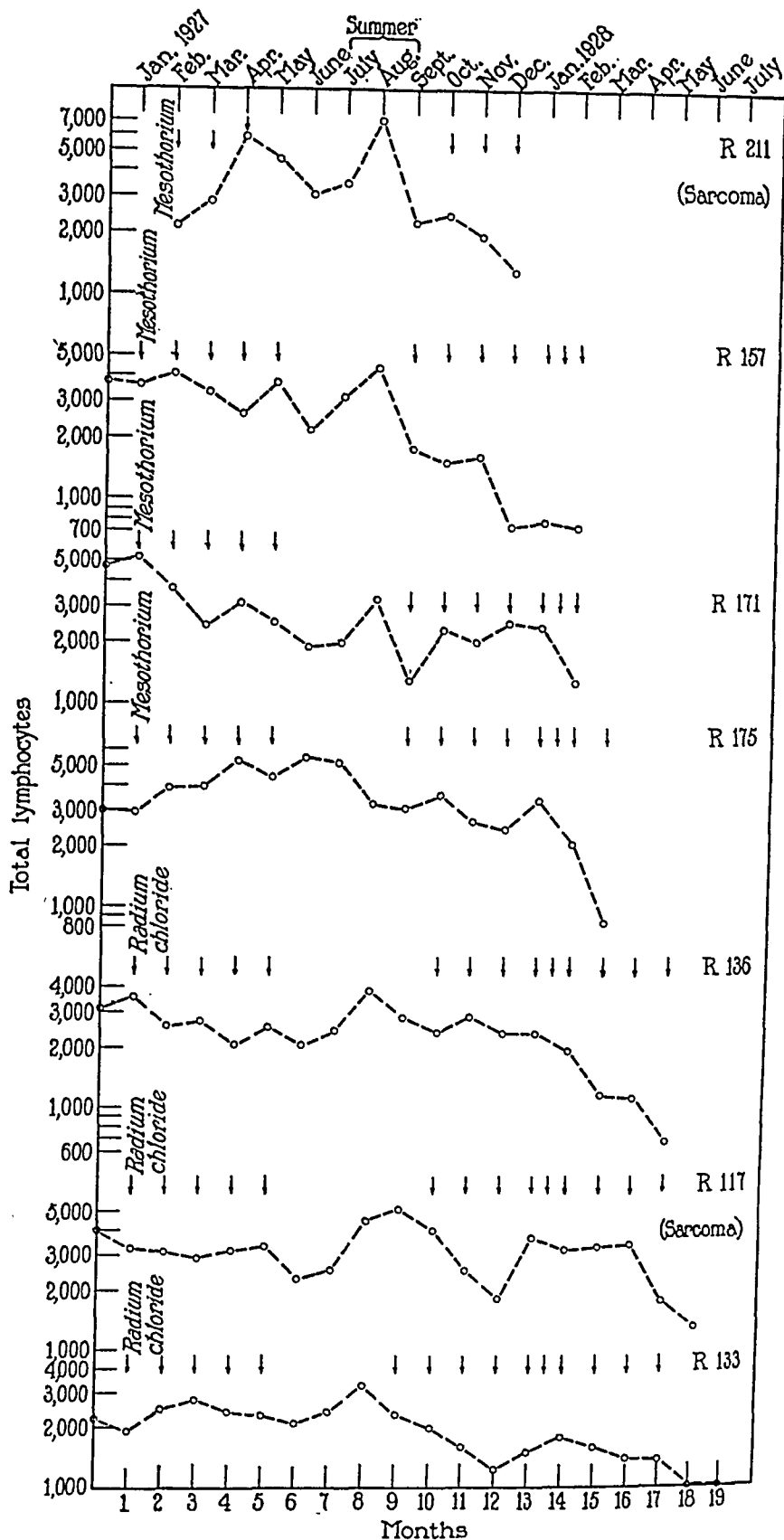
(1). In these experiments the dose of mesothorium was higher than of radium chloride in the proportion of 7.7 to 5.1 which in terms of alpha particles means a ratio of 38 to 20. In considering the biological effect of radium it is necessary to know the amount stored in the tissues, since a considerable amount of the radium given is excreted (49). Methods for measuring radioactive materials in the living person have been developed by Flinn *et al.* (49-52).

#### *Effects on Lymph Nodes and Lymphocytes*

The effects on the lymph nodes in our series can be followed in Figs. 1 to 6, and they are to be compared with the changes in the lymphocytes shown in Graph 2. In general it will be noted that the lymphocytes fell faster after mesothorium than after radium chloride. After three injections of 5.1 micrograms of radium chloride (R 210), only the peripheral lymph nodes were affected, indicating that they are more sensitive to radioactive materials than the mesenteric nodes and spleen. They showed a uniform hyperplasia of the primitive stem cells (Fig. 1) with extremely active cell division.

By the end of 11 months after six injections of 7.7 micrograms of mesothorium (R 211) all of the lymphoid tissues were involved. The animal still showed the increase in the primitive cells, but, in contrast to the previous experiment, there was no cell division. Instead, large areas of the primitive cells showed a peculiar type of degeneration in which the changes that could be detected were in the chromatin of the nuclei (Fig. 6). The sections look as if the chromatin had dissolved and diffused through the nuclear membrane into the cytoplasm. The same type of cellular damage involved all of the cells of the thymus. This was the only animal in which we found damage to the stem cells in the stage of their hyperplasia; in the other animals a similar damage was seen to the stem cells scattered in their normal proportions in the follicles. This difference will be clear by comparing Figs. 2 and 4.

The six remaining animals were allowed to live from 14 to 19 months after the first injection. The study of their tissues shows that after the period of stimulation to the primitive stem cell, there was a marked development of immature lymphocytes, the type of cells with deeply basophilic cytoplasm, known as the lymphoblast. In the follicles



GRAPH 2

this was shown by a marked increase in the proportion of the large lymphoblasts to the mature small lymphocytes as seen in Fig. 5, while in many of the lymph cords there was a complete replacement of mature lymphocytes by lymphoblasts.

This condition was both accompanied and followed by signs of damage to the stem cell of the lymphocytes, illustrated by the cells with arrows in Figs. 4 and 6. This damage was a shrinkage of the nuclei, as can be seen by comparing with the corresponding normal nuclei in Fig. 2, and with a gradual depletion of the chromatin until nothing stainable was left in the nuclei but an inner rim to the nuclear membrane. No such signs of damage could be made out either in the lymphoblasts or in the mature lymphocytes.

The next stage in the changes of the lymph nodes was depletion, starting with the cells of the lymph cords and gradually involving the follicles as well. The contrast between the period of atrophy and the early stimulation is shown in Figs. 1 and 3. A node in which all of the lymph cords had disappeared looked cystic in the gross specimen; the process went on to the complete atrophy of some nodes. These processes were not all in the same stage in the different nodes of an animal. For example, in R 133 the follicles of the spleen showed a hyperplasia of the stem cell while the lymph nodes showed atrophy. In general the depletion was more extreme in the peripheral nodes than in the mesenteric nodes and in the spleen; in some instances the lymphoid tissues may thus be summed up in three stages: first, a period of stimulation to the stem cell; second, a shift to the immature stage known as the lymphoblast; and third, a depletion of the nodes due to such a damage of the stem cell that the losses in lymphocytes could no longer be made up.

With these processes in mind it is interesting to follow the changes in the lymphocytes in the blood. The blood cells of the animal which developed the abscess and was killed after three injections are shown in Graph 1. We do not know the reason for the rise of all of the strains of white blood cells, unaccompanied by a rise in red cells, which is shown for April 21; the curve of the red cells indicated that it was not a process of dehydration. However, the subsequent curve of the lymphocytes shows that there was no sustained rise in circulating



lymphocytes in this animal to reflect the hyperplasia of the primitive stem cells shown in Fig. 1.

In Graph 2 it will be seen that during the initial 5 months in which the primitive cells of the lymph nodes were stimulated, three animals show a rise in lymphocytes, three a fall, and one no change. Thus there was no constant early reaction of the lymphocytes in the blood which may have been due to variations in the condition of the different lymph nodes.

All of the graphs show a rise in lymphocytes during the summer months. There are two factors to be considered in relation to the rise in lymphocytes in summer; for July and August the differential counts were made on fixed films and it is our experience that lymphocytes run on an average 4 per cent higher in counts made with fixed films than in counts of the living cells (68). In every instance, the first counts of lymphocytes, taken before the second series of injections was started, were lower than the counts in August. These points make the rise during the summer months of less significance as an effect of the injections of radioactive material.

Every graph shows that there was a final, significant, and steady fall in lymphocytes covering in general the period of the second series of injections. The study of the tissues for each animal has shown that the lymph nodes finally exhibited a marked shift to the lymphoblast and a depletion of the nodes due to a failure of the stem cells to replace lymphocytes.

The final period of the more precipitous fall in lymphocytes, covering the 3 or 4 months before each animal was killed, has been in each instance correlated with a loss in weight in the animals. They all showed a gain in weight during the early months of the experiment, probably due to the better feeding of the animals which were all bought from dealers and not bred in the laboratory. Four were females and of them three gave birth to young during the experiment. The loss in weight which is associated with the period of depletion of the lymph nodes is of great significance in connection with the studies of the relation of lymphocytes to resistance to cancer and to tuberculosis (9-26, 69, 70). Thomas (71) has found in a study of a series of tuberculous rabbits that in the final period of broken resistance there is a loss in weight which is proportional to the fall in lymphocytes.

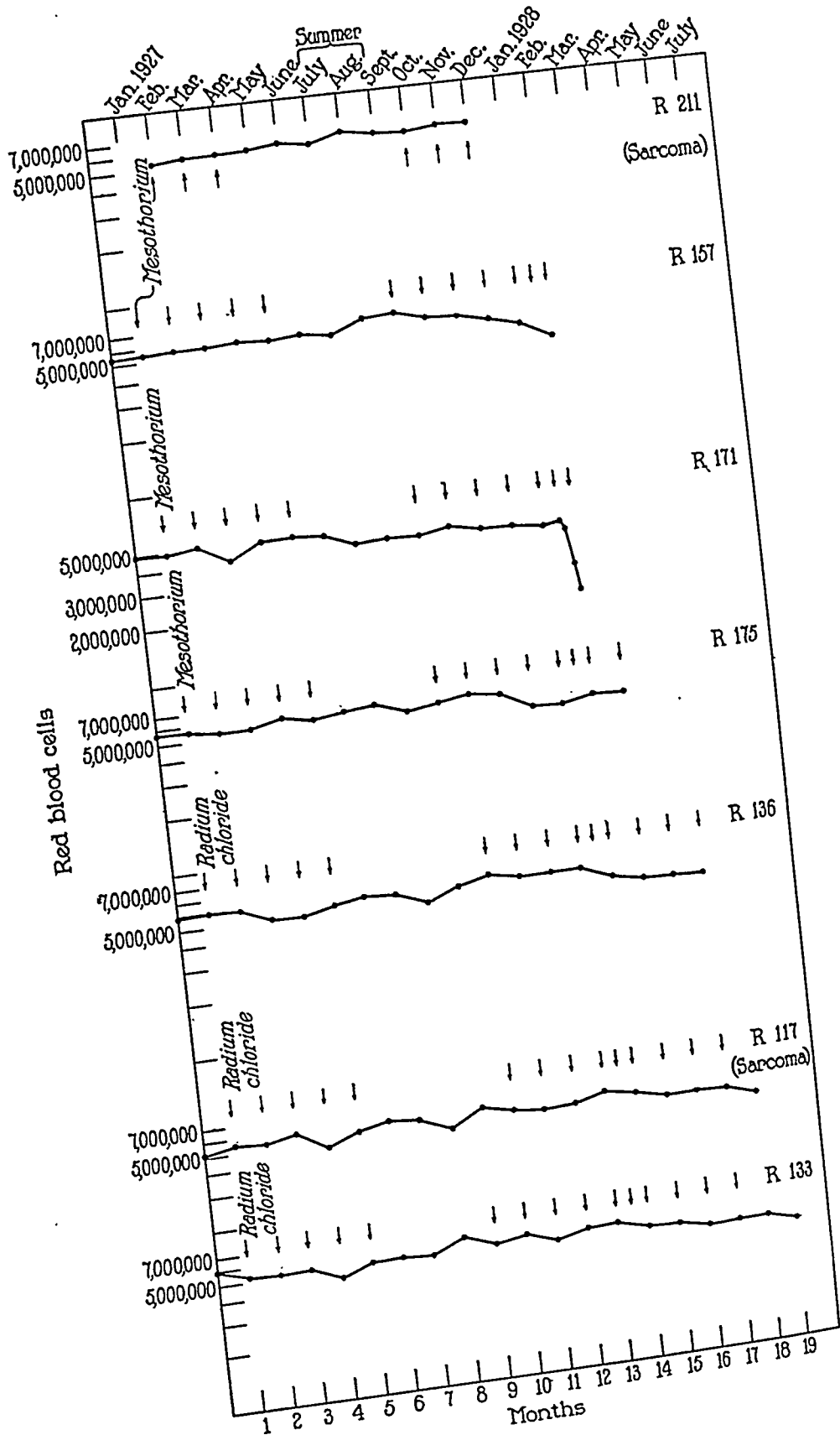
This series of radiated animals demonstrates the same phenomenon,—an atrophy of the lymph nodes, a lymphopenia, and at the same time a loss in weight. This correlation was constant and significant.

*Effects on the Bone Marrow and the Red Cells*

There were no changes in the neutrophilic leucocytes in this series other than those associated with the development of the abscesses. There were also no changes in eosinophilic leucocytes and in monocytes, but in every instance there was a fall in basophils during the period of the final fall in lymphocytes.

The fact that the bone marrow as a hematopoietic organ is less sensitive than the lymph nodes is shown not only by the lack of change in granulocytes but also by the slight changes in the red cells. This will be clear by comparing the graphs of the red cells (Graph 3) with those of the lymphocytes in Graph 2. There was no change whatever in the color index throughout the experiments, even in R 171, at the time of the precipitous fall in red cells. Rabbit R 211, which was killed in 11 months, showed no anemia whatever; the original count of red cells was 5,200,000 and the final count was 6,000,000.

It will be noted on Graph 3 that all of the animals except the first two showed a slight fall in red blood cells by March or April, but all except R 171 showed an excellent recovery to the level of 6,000,000 cells by August or the early months of the fall. All of the animals which received the extra injection in January showed then a progressive fall in red cells. The graph of R 171 shows that this animal was a special case in regard to the red blood cells; the original level of the red cells in this animal was lower than in the others and, from July on, there was a slight loss of red cells which became extreme after the extra injection in January. All of the figures on this chart are averages except the records shown for February for R 171, in which the 4 counts for the first half of the month are plotted separately in order to bring out better the rapidity and severity of the fall in red cells from 4,380,000 to 1,800,000. In the preceding months the counts of the red cells did not vary much from the average. The signs were of a peripheral destruction of red cells: marked fragmentation of these cells, as seen in the supravital preparations, and the extreme increase in iron-containing pigment in spleen and bone marrow seen



GRAPH 3

in sections. However, the bone marrow also showed greater damage than any of the other animals in the series, for it not only revealed the change in the fat cells and the marked mottling of active areas and zones of complete aplasia, but in the active areas there were some large zones of immature erythroblastic cells comparable to the great increase in lymphoblasts described in the lymph nodes. This is interesting in connection with the reports of the occurrence of a primary anemia due to exposure to radioactive substances in human cases (27, 38-43).

All of the other animals in the series, except the three that were killed too early (R 134, R 210, and R 211), showed changes in the fat cells and the mottling of the marrow but not the shift to early erythroblastic or megaloblastic marrow. The mottling of the marrow is due to a change in the normal distribution of active and inactive marrow; in the normal rabbit there are large areas, often at the periphery, of active marrow and smaller, centrally placed, inactive zones. The mottling is a change in this pattern. It is easily seen with the unaided eye and may indicate a local damage to the stem cells of the marrow, due to an uneven distribution of the radioactive material in the bone. The great reserve in the tissues of the marrow may account for the fact that so much change in the marrow was not reflected to a greater extent in the blood. From the results on Graph 3, it is likely that the first series of injections of the radioactive material caused enough peripheral destruction of the red cells to give rise to a slight anemia but that there was not enough damage to the bone marrow to be detected in the blood (R 211). With the second series, however, the crowding of the two injections into the 1 month caused not only an increased peripheral destruction but also instigated changes in the bone marrow which were progressive. In one animal in the series (R 171) both the red cells and the bone marrow were more susceptible to radioactive material than in the other animals, and in this instance there occurred both extreme destruction of mature red cells and the early signs of the changes in the marrow of the increased proportion of immature cells of the erythroid series which eventually results in an anemia of the primary type. Thus, the effects on the red cells were similar in type but less in degree than those on the lymph nodes. They are, increased destruction of red cells, a shift to the immature phases in the marrow, and finally aplasia.

*Effects on Thymus and Liver*

The observations on the thymus in our series are meager, but they suggest that the cells of the thymus (R 211) are affected in the same manner as the primitive stem cells of the lymph nodes and that they are not as resistant to radioactive material as mature lymphocytes. The end-result of this effect is a complete atrophy of the organ. A damage to the liver has been noted in four animals; with mesothorium (three animals) there were changes in the nuclei, similar in kind but less in amount than those of the stem cell of the lymph nodes; namely, a loss of chromatin from the nucleus into the cytoplasm. The damage was reflected in a marked reduction in the size of this organ. Both of the animals which showed paralysis—one of them certainly without damage of the central nervous system—exhibited the reduction in size of the liver. These symptoms are possibly explained by the demonstration by Mann (72) of weakness and paralysis in dogs after removal of the liver and of the same phenomena in rabbits after partial liver insufficiency by McMaster and Drury (73, 74) and after total removal of the liver by Drury (75).

*Production of Osteogenic Sarcomata through Storage of Radioactive Material in the Bones*

Certain results in our series of animals are interesting in relation to the industrial hazard in the use of radioactive materials: first, the number of abscesses that developed; second, the spontaneous fracture; and third, the development of osteogenic sarcomata in two animals. Our percentage of tumor formation, two out of seven animals, was high in comparison with the figures of Daels *et al.* It has been demonstrated that the bones store the radioactive material. The sarcomata were clearly derived from osteoblasts; in one instance there was newly formed bone associated with giant cells of the type of the osteoclast. In one case there were metastases. We have lacked one element in the proof of malignancy; namely, successful transplantation of the tumors. No attempts in this direction were made.

## SUMMARY

The observations in this work suggest that with certain doses of radioactive material, the fundamental damage in the lymphoid tissues

is to the stem cell and that the damage is to the chromatin of the nuclei of these cells. The erythroid tissues are apparently less susceptible to radioactive material than the lymphoid tissues but an original anemia of secondary type from peripheral destruction may eventually be changed to one of primary type through decreased maturation of primitive cells in the marrow. The damage of lymph nodes and bone marrow leads to atrophy of these organs. The cells of the liver and thymus suffer nuclear damage of the same general character as is seen in the lymph nodes, and there is an atrophy of these organs. The storage of the radioactive material in the bones gave rise to osteogenic sarcomata in two out of seven rabbits surviving from 11 to 19 months. A repetition of the experiment has been undertaken with more intensive studies to test the validity of the findings.

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EXPLANATION OF PLATES  
PLATE 16

FIG. 1. Peripheral lymph node of Rabbit R 210, killed 17 days after the third intravenous injection of 5.1 micrograms of radium chloride, to show the degree of hyperplasia of the pale cells of the follicles. Stained in hematoxylin and eosin.  $\times 50$ .

FIG. 2. Center of one of the peripheral follicles of the same node as in Fig. 1, to show the predominance of large pale cells and the number of them in division. Stained in hematoxylin and eosin.  $\times 1,000$ .

FIG. 3. Cervical lymph node of Rabbit R 136, killed 22 days after the fourteenth intravenous injection of 5.1 micrograms of radium chloride, to show the stage of depletion of the nodes. Stained in hematoxylin and eosin.  $\times 50$ .

FIG. 4. Mesenteric lymph node from Rabbit R 157, killed 5 days after the twelfth intravenous injection of 7.7 micrograms of mesothorium, to show the damage to the stem cells, which are indicated by arrow. The normal small lymphocytes are printed very dark in order to bring out the pale nuclei of the stem cells. Stained in hematoxylin and eosin.  $\times 1,000$ .

FIG. 5. Peripheral lymph node of Rabbit R 171, killed 15 days after the twelfth injection of 7.7 micrograms of mesothorium to show the decrease in mature lymphocytes and the increase in lymphoblasts in a peripheral follicle. Stained in Giemsa.  $\times 270$ .

FIG. 6. Mesenteric lymph node of Rabbit R 211, killed 22 days after the sixth injection of 7.7 micrograms of mesothorium, to show the damage to masses of the stem cells in a peripheral follicle. The small dark nuclei are of normal small lymphocytes. Stained in hematoxylin and eosin.  $\times 1,000$ .

#### PLATE 17

FIG. 7. Sarcoma in the bone marrow of the left humerus of Rabbit R 117, which was killed 41 days after the fourteenth injection of 5.1 micrograms of radium chloride. The left half of the figure is the sarcoma; the right half shows the zone of invasion of the bone marrow by the tumor cells, with more normal bone marrow at the extreme right. Stained in hematoxylin and eosin.  $\times 120$ .

FIG. 8. Section from the center of the sarcoma seen in Fig. 7, to show the character of the tumor cells, one of which is in division. Stained in hematoxylin and eosin.  $\times 1,000$ .

FIG. 9. Section through the edge of the sarcoma seen in Fig. 7, to show the tumor cells, marked by arrows, invading the marrow between masses of myelocytes, leucocytes, and megalokaryocytes. Stained in hematoxylin and eosin.  $\times 1,000$ .

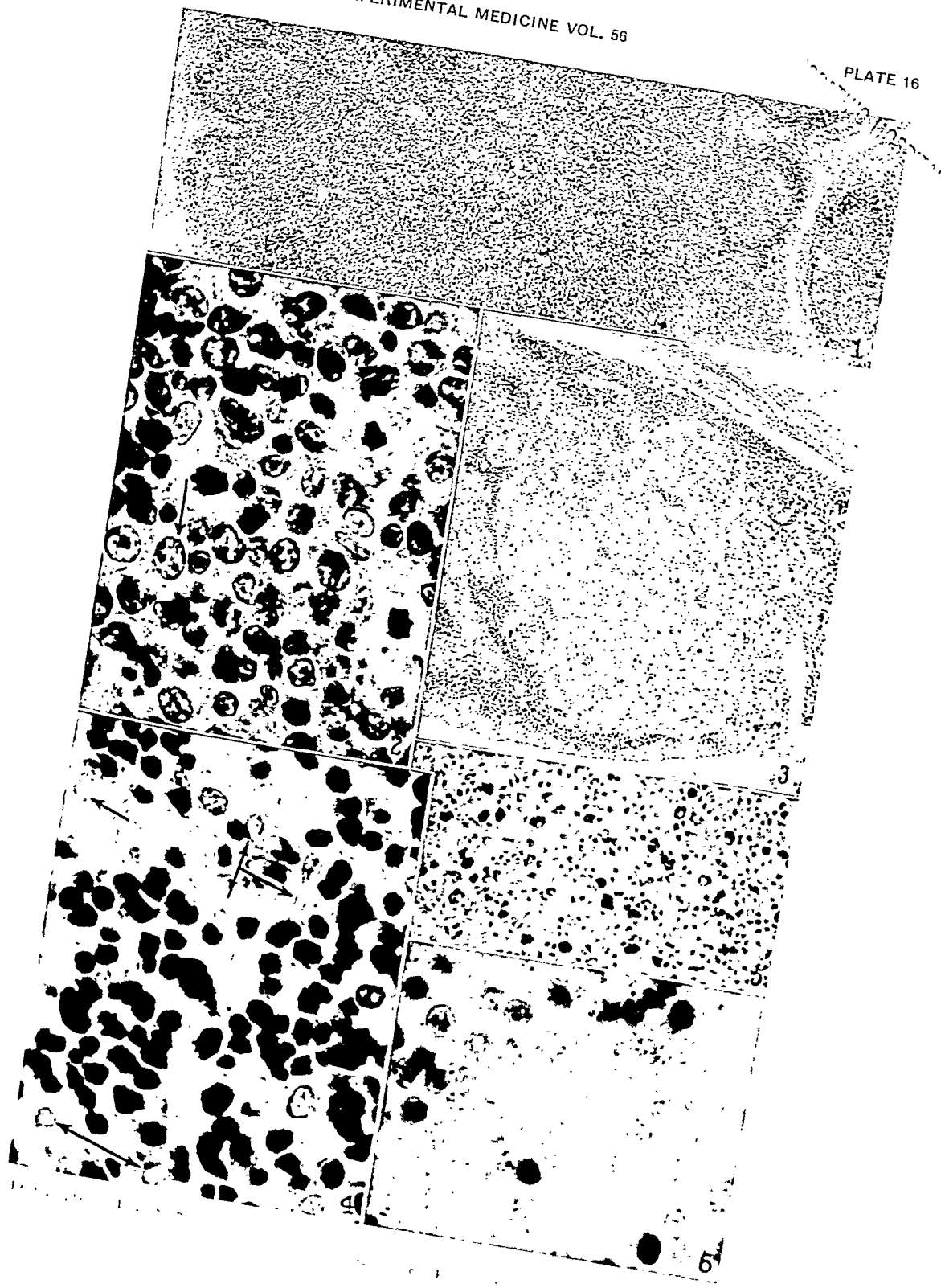
FIG. 10. Section of the lung of Rabbit R 157, killed after the twelfth intravenous injection of mesothorium, to show the complete absence of air sacs and the proliferation of bronchial epithelium due to a chronic inflammatory process. Arrow A—bronchus; Arrow B—membrane of bronchial epithelium. Stained in hematoxylin and eosin.  $\times 200$ .

#### PLATE 18

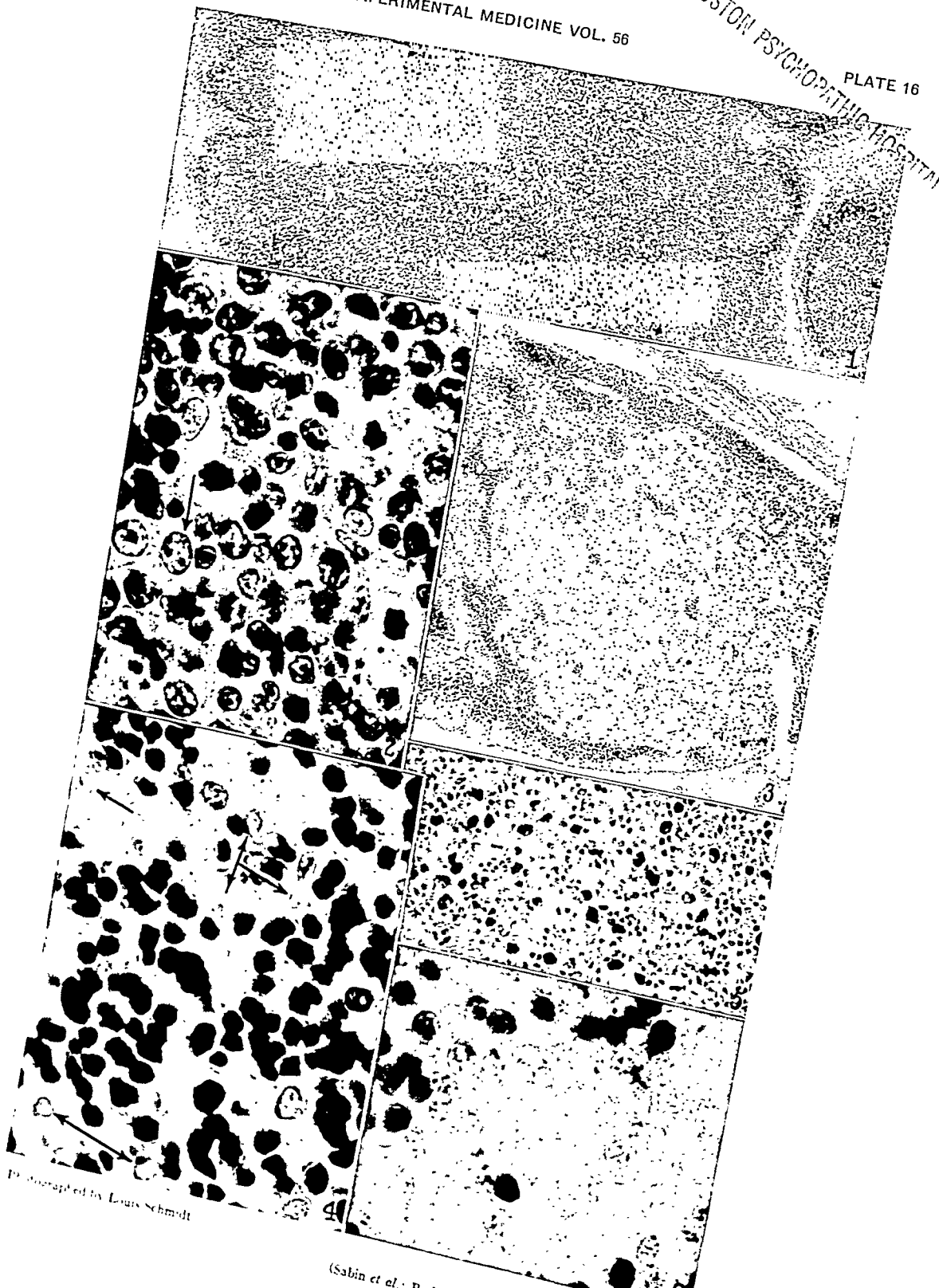
FIG. 11. Section of a sarcoma which almost completely replaced the bone marrow of the right femur of Rabbit R 211, killed 22 days after the sixth injection of 5.1 micrograms of radium chloride. Stained in hematoxylin and eosin.  $\times 120$ .

- FIG. 12. Section of the tumor, shown in Fig. 11, to show the newly formed bone, arrow A, and the giant cell, Arrow B, of the type of osteoclast near the bone. Stained in hematoxylin and eosin.  $\times 1,000$ .
- FIG. 13. Section of bone from Rabbit R 211, near the edge of a sarcoma of the bone marrow. The edge of the bone is denuded of osteoblasts, but they may be represented by the three large tumor cells nearby. Stained in hematoxylin and eosin.  $\times 1,000$ .
- FIG. 14. Damaged bone near the place of fracture from the right femur of Rabbit R 211 to show the complete freeing of the bone corpuscles from their lacunuli and the fragmentation of the bony matrix. Stained in hematoxylin and eosin.  $\times 270$ .







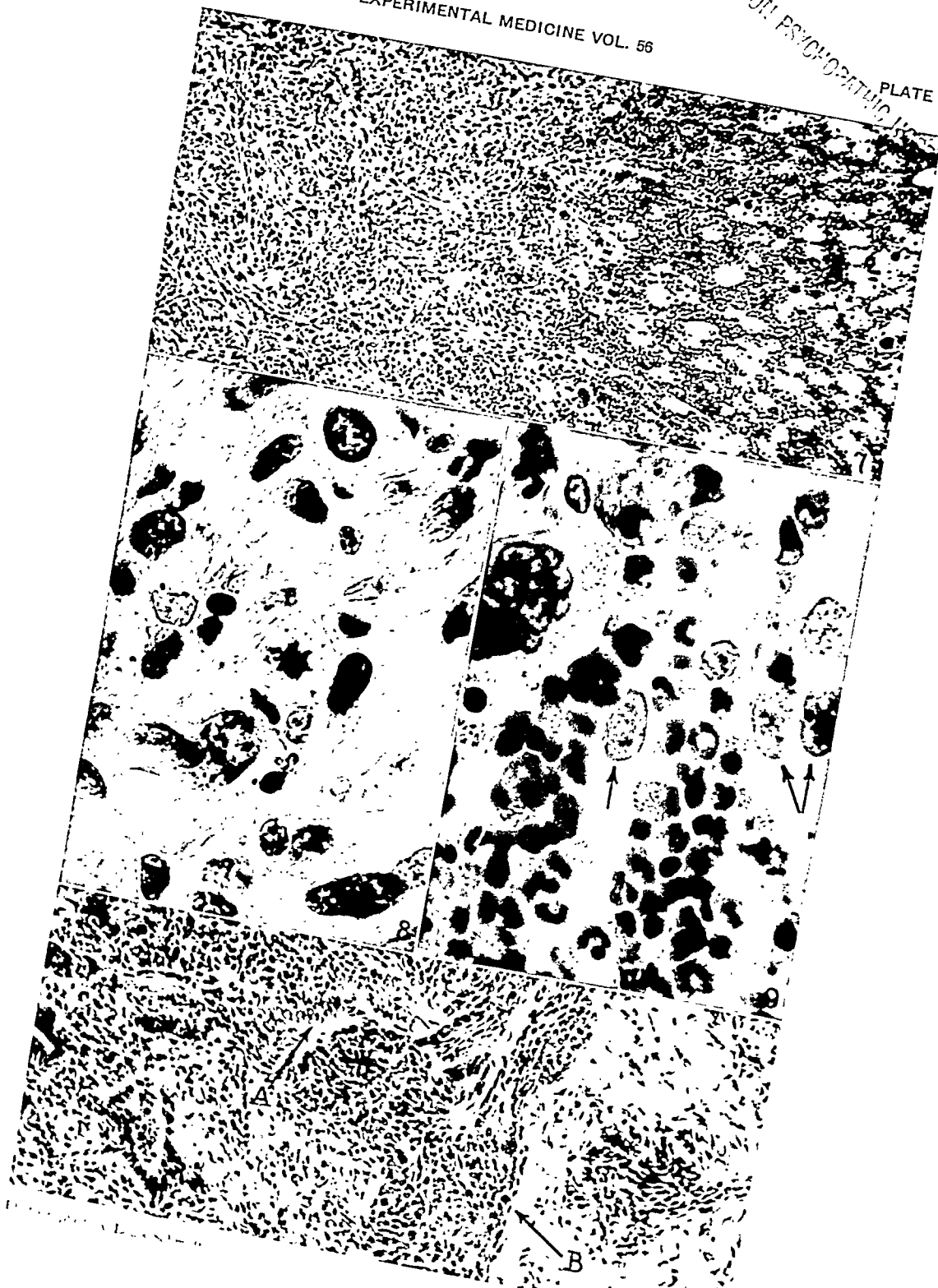


Photographed by Louis Schmidt

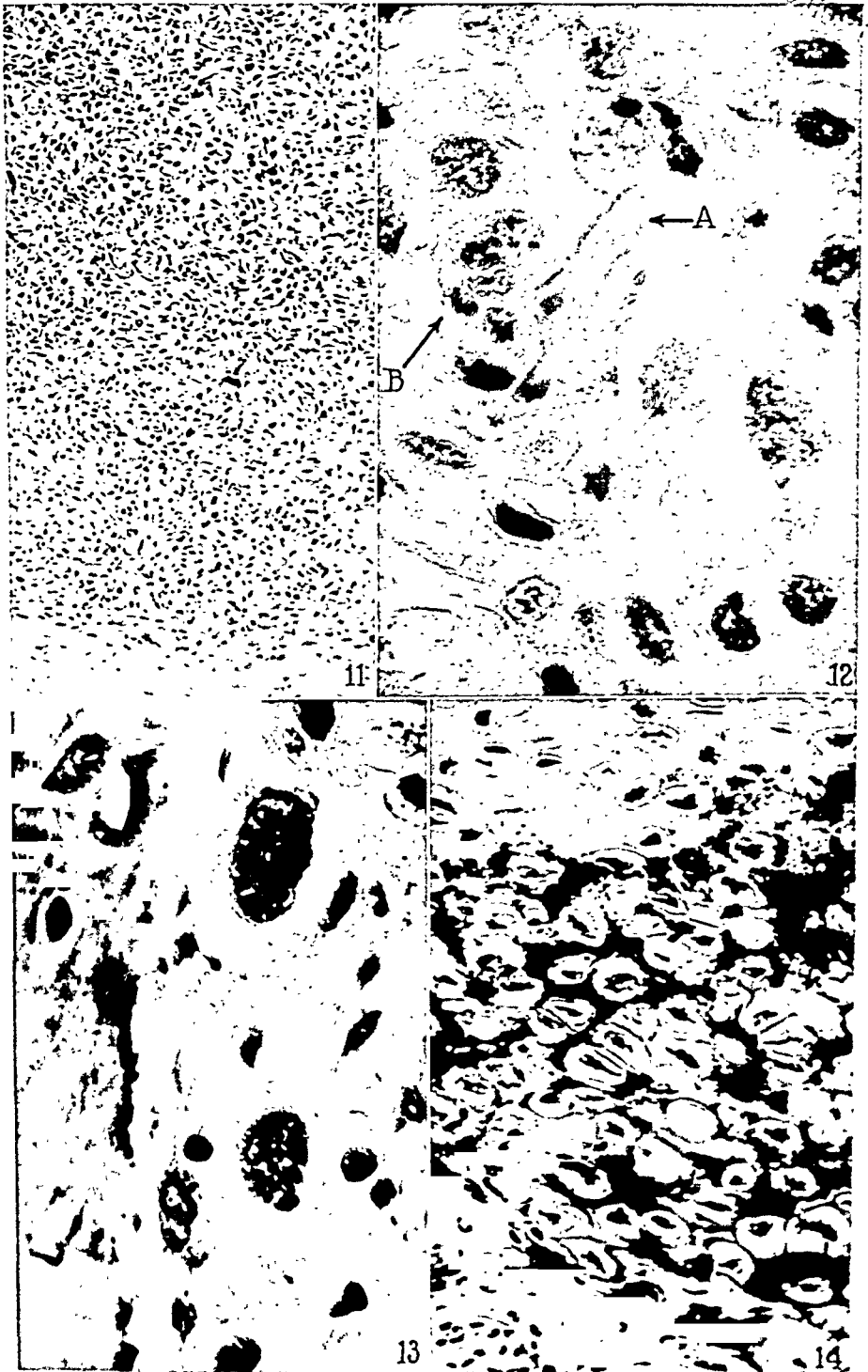
(Sabin et al., 1923)





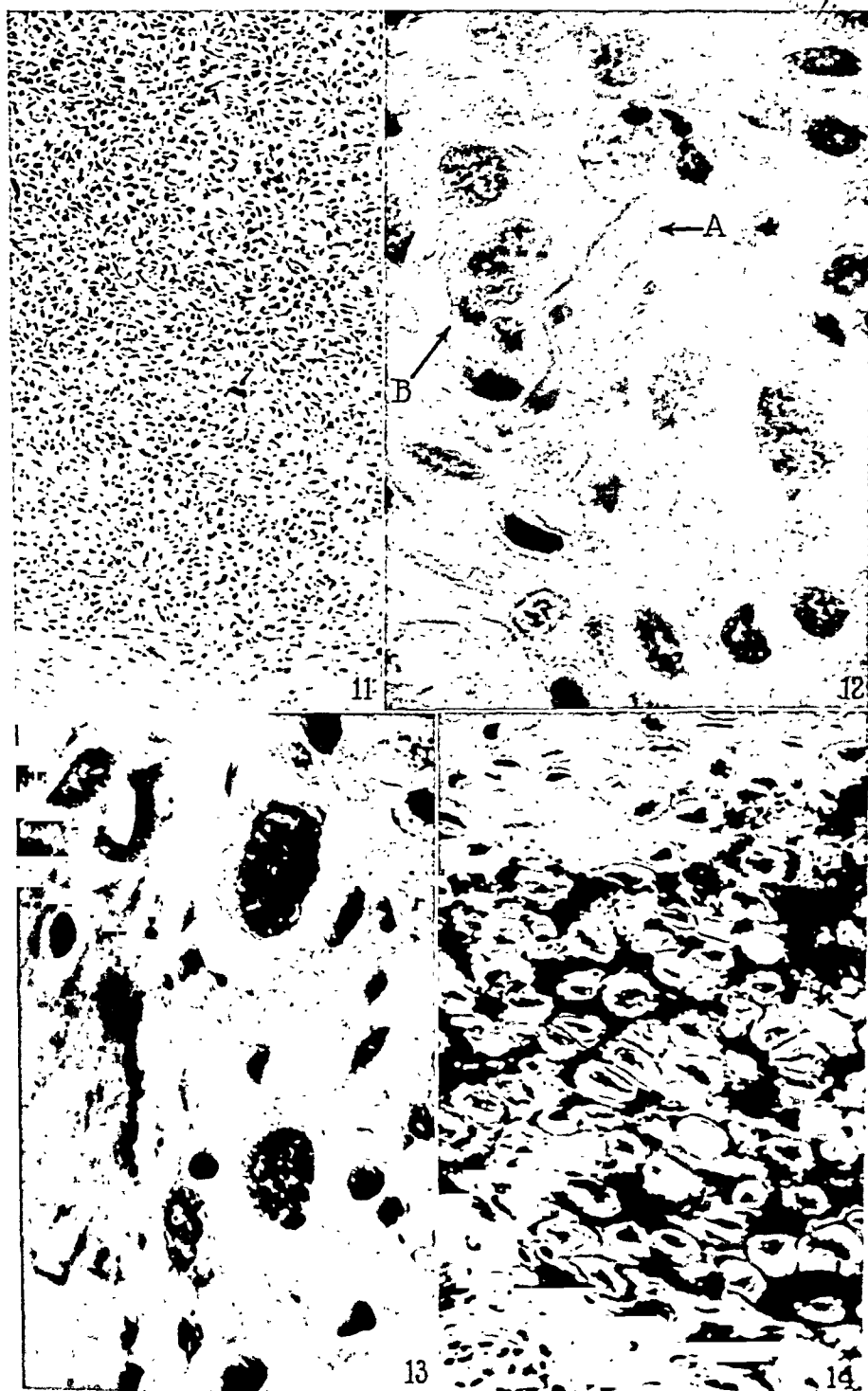






Photographed by Louis Schmitt





Photographed by Louis Schmitt



# FURTHER STUDIES ON THE NATURE OF THE PHENOMENON OF LOCAL SKIN REACTIVITY TO BACTERIAL FILTRATES: TOXIC FACTORS DERIVED FROM THE BLOOD SERUM

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As reported previously (1) the *sine qua non* of the phenomenon of local skin reactivity to bacterial filtrates is that the second injection be given *via* the blood stream. Whilst large doses reinjected locally remain ineffective, an amazingly small quantity of toxic filtrate may elicit severe hemorrhagic necrosis at the prepared skin site (*i.e.*, with some batches of meningococcus filtrates less than 0.000009 cc., per kilo of body weight).

Inasmuch as these observations suggest that there occurs some interaction between the toxic substances injected and the blood prior to the elicitation of the reaction, it was of interest to determine whether a disturbance in the colloidal state of the blood would bring about formation of reacting factors. The results of these studies are embodied in the present paper.

## EXPERIMENTAL

### I

*Testing of Sera for Skin-Preparatory Factors.*—The abdominal skin of rabbits was prepared by single or four simultaneous injections of sera tested. A dose of 0.25 cc. was used for each intradermal injection. 24 hours later the rabbits received each a single intravenous injection of *B. typhosus* culture filtrate, in a dose of 2 cc., per kilo of body weight. Reactions were read 4 to 5 hours later. Rabbits succumbing within 4 hours after the intravenous injection were not recorded. The *B. typhosus* culture filtrates were made according to methods previously described (2). The results are summarized in Table I.

As is seen from Table I, one batch of crude commercial antityphoid horse serum and several batches of chemically concentrated immune



sera showed skin-preparatory potency. However, the antityphoid horse serum was stored in these laboratories for 2 years and, although it was found sterile at the time of the tests, it might have been con-

TABLE I  
*Sera Tested for Skin-Preparatory Factors*

Sera injected intradermally	Total No. of areas tested	No. of negative reactions	No. of doubtful reactions	No. of positive reactions
Comm. antityphoid horse serum.....	12	0	0	12
3 antityphoid rabbit sera* 90, 78, 59.....	32	32	0	0
2 batches of pooled normal rabbit sera.....	36	36	0	0
Pooled sera of rabbits injected with typhoid fltr. kwt. immediately before bl.....	4	0	0	4
Pooled sera of rabbits injected with typhoid fltr. 3 cc. kwt. 24 hrs. before bl.....	16	16	0	0
Serum of rabbit injected with <i>coli</i> fltr. 3 cc. kwt. 4 hrs. before bl.....	12	12	0	0
Serum of rabbit injected with typhoid fltr. 3 cc. kwt. 3 hrs. before bl.....	12	12	0	0
Serum of rabbit injected with typhoid fltr. 2 cc. kwt. twice 24 hr. interval; bleeding 2 hrs. after 2nd injection.....	16	16	0	0
7 antityphoid goat sera*.....	28	27	1	0
Mount Sinai antityphoid sera of Horses* 2, 3, 4, 5 and 6.....	20	20	0	0
B. H. typhoid diagnostic serum.....	16	16	0	0
2 batches anti- <i>shigae</i> rabbit serum.....	24	24	0	0
2nd wk. typhoid pt. serum.....	12	12	0	0
23 batches comm. antimeningococcus horse serum.....	180	178	2	0
2 diagnostic B. H. Pneumococcus <sub>3</sub> sera.....	20	20	0	0
B. H. therapeutic Pneumococcus <sub>2</sub> Serum 930.....	16	16	0	0
B. H. therapeutic Pneumococcus <sub>2</sub> Serum 28/934.....	20	0	0	20
B. H. therapeutic Pneumococcus <sub>3</sub> serum.....	20	4	0	16
Comm. antimening. conc. serum.....	8	4	0	4
Mount Sinai antityphoid conc. horse serum..	16	0	00	16

kwt. = per kilo of body weight. B.H. = New York Board of Health. bl. = bleeding. fltr. = filtrate. pt. = patient. comm. = commercial. conc. = concentrated.

\* Bleedings obtained at various stages of immunization were selected for these tests.

taminated sometime during its prolonged storage and resterilized by the preservative. It is also clear that chemically concentrated sera are grossly contaminated during the treatment. As a matter of fact, the Mount Sinai antityphoid concentrated serum prepared in these laboratories (3) when cultured a number of times during the process of concentration proved consistently to be heavily contaminated by a variety of microorganisms (*i.e.*, *B. proteus*, *coli*, *subtilis*, staphylococcus, etc.) prior to the routine addition of 0.6 per cent tricresol and ether mixture. The majority of these bacteria are capable of producing toxic substances of high skin-preparatory potency (4). Moreover, the rabbit serum obtained by heart puncture immediately after intravenous injection of as much as 4 cc. of *B. typhosus* culture filtrate, per kilo of body weight, also possessed a definite skin-preparatory potency. On the other hand, no skin-preparatory factors were found in a great variety of normal sera, immune sera of various animals obtained during different stages of immunization and sera of rabbits injected with large doses of toxic filtrates some hours before bleeding, all of which were collected under sterile precautions and properly preserved.

*Testing of Sera for Reacting Factors.*—Rabbits were prepared by one intradermal injection of 0.25 cc. of a potent *B. typhosus* "agar washings" filtrate (2). 24 hours later they received each a single intravenous injection of the serum tested. The reactions were read 4 to 5 hours later. Each serum was tested in a group of three rabbits, unless stated otherwise.

The following sera tested in doses per kilo of body weight, indicated below, were found totally devoid of reacting potency.

(1) Normal human serum, 1 cc.; (2) antimeningococcus horse Serum H<sub>7</sub>Bl<sub>405</sub>, 3 cc.; (3) antimeningococcus rabbit Serum S<sub>10</sub>, 1 cc.; (4) antimeningococcus rabbit Serum S<sub>215</sub>, 1 cc.; (5) antimeningococcus horse Serum H<sub>7</sub>Bl<sub>225</sub>, 2 cc.; (6) normal rabbit serum, 2 cc.; (7) normal guinea pig serum, 2 cc.; (8) normal rat serum, 2.5 cc. of 1:10 dilution; (9) normal chicken serum, 1 cc.; (10) antityphoid immune chicken serum, 1 cc.; and (11) pooled serum of rabbits injected intravenously with 2.5 cc. of *B. typhosus* culture filtrate, per kilo of body weight, 3 hours before bleeding, 5 cc. (tested in four rabbits).

The commercial antityphoid horse serum above referred to which

The results which represent readings of three surviving rabbits for each preparation tested, were entirely negative.

### *Testing of Precipitates for Reacting Factors*

Each rabbit received a single intradermal injection of 0.25 cc. of undiluted *B. typhosus* "agar washings" filtrate and 24 hours later an intravenous injection of 1 cc. of the suspension of precipitate tested, per kilo of body weight. There were no deaths following these injections. The results are recorded in Table II. In

TABLE II  
*Reacting Potency of Various Sera Precipitates*

Precipitinogen-containing serum	Precipitating antiserum	Proportions of precipitinogen-containing serum and precipitating antiserum in mixtures	Results with precipitates		
			Total No. rabbits	Positive rabbits	Negative rabbits
Normal horse serum	Anti-horse rabbit Serum R I	0.33 cc. + 0.66 cc.	3	3	0
" " "	Anti-horse rabbit Serum R II	0.33 " + 0.66 "	3	2	1
" " "	Anti-horse rabbit Serum R III	0.66 " + 0.33 "	3	2	1
Comm. antimeningococcus horse Serum M <sub>18</sub>	Anti-horse rabbit Serum R <sub>357</sub>	0.66 " + 0.33 "	3	3	0
Mount Sinai antityphoid horse Serum H <sub>4</sub> Bl <sub>232</sub>	" "	0.66 " + 0.33 "	3	2	1
Mount Sinai antityphoid horse Serum H <sub>4</sub> Bl <sub>196</sub>	Anti-horse goat Serum G <sub>165</sub>	1 cc. + 1 cc. diluted 1:5	3	2	1
Mount Sinai anti- <i>coli</i> horse Serum H <sub>60</sub> Bl <sub>183</sub>	Anti-horse rabbit Serum R <sub>353</sub>	0.66 cc. + 0.33 cc.	3	3	0

comm. = commercial.

this table, the expression "positive rabbit" means that there was obtained in the rabbit's prepared skin site a severe hemorrhagic and necrotic lesion 4 to 5 hours after the intravenous injection. The lesions, which were intense in the majority of positive rabbits, were characteristic of the phenomenon of local skin reactivity to bacterial filtrates (6). By "negative rabbit" is meant absence of skin reaction following the intravenous injection.

As is seen from Table II, suspensions of precipitates derived from mixtures of precipitinogen-containing serum with precipitating anti-

serum possess a high reacting potency. The active preparations can be obtained from combinations of some normal animal sera with homologous antisera, as well as from combinations of sera of animals immunized with bacterial antigens with antisera against normal sera of the same animal species.

### *Titration of Reacting Potency of Precipitates*

In these experiments, the precipitates were derived from mixtures of anti-meningococcus anti-human horse sera with normal human sera, in proportions of

TABLE III  
*Titration of Reacting Potency of Serum Precipitates*

Date of preparation	Precipitinogen-containing serum	Precipitating antiserum	Dilution of precipitate suspension	Total No. of rabbits tested	No. of negative rabbits	No. of doubtful rabbits	No. of positive rabbits
May 8, 1931	Pooled normal human sera	Antimeningococcus anti-human Serum H <sub>7</sub> Bl <sub>22</sub>	Undiluted	3	1	0	2
" 8, 1931	" "	" "	1:10	3	1	0	2
" 8, 1931	" "	" "	1:50	3	2	0	1
" 8, 1931	" "	" "	1:100	3	3	0	0
June 20, 1931	Normal human serum	" "	Undiluted	3	2	1	0
" 20, 1931	" "	" "	1:5	3	3	0	0
July 23, 1931	" "	" "	Undiluted	3	2	0	1
" 23, 1931	" "	" "	1:5	3	3	0	0
" 29, 1931	" "	Antimeningococcus anti-human horse Serum H <sub>7</sub> Bl <sub>155</sub>	Undiluted	3	0	0	3
" 29, 1931	" "	" "	1:5	3	3	0	0

0.9 to 0.05 cc., respectively. The precipitates obtained in the manner described above gave highly turbid suspensions and had a tendency to settle on the bottom of the test-tube. The aggregates were easily broken up by shaking. The dilutions 1:5 and 1:10 of these suspensions were also quite turbid. In dilution 1:50 the turbidity was slight and in dilution 1:100 the fluid was clear. Various dilutions of the suspensions were tested for reacting potency in rabbits prepared with *B. typhosus* "agar washings" filtrate. The tests were made in the course of the week following the preparation of the suspensions. The results are reported in Table III.

The results which represent readings of three surviving rabbits for each preparation tested, were entirely negative.

### *Testing of Precipitates for Reacting Factors*

Each rabbit received a single intradermal injection of 0.25 cc. of undiluted *B. typhosus* "agar washings" filtrate and 24 hours later an intravenous injection of 1 cc. of the suspension of precipitate tested, per kilo of body weight. There were no deaths following these injections. The results are recorded in Table II. In

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			Total No. rabbits	Positive rabbits	Negative rabbits
Normal horse serum	Anti-horse rabbit Serum R I	0.33 cc. + 0.66 cc.	3	3	0
" " "	Anti-horse rabbit Serum R II	0.33 " + 0.66 "	3	2	1
" " "	Anti-horse rabbit Serum R III	0.66 " + 0.33 "	3	2	1
Comm. antimeningococcus horse Serum M <sub>18</sub>	Anti-horse rabbit Serum R <sub>357</sub>	0.66 " + 0.33 "	3	3	0
Mount Sinai antityphoid horse Serum H <sub>4</sub> Bl <sub>232</sub>	" "	0.66 " + 0.33 "	3	2	1
Mount Sinai antityphoid horse Serum H <sub>4</sub> Bl <sub>196</sub>	Anti-horse goat Serum G <sub>165</sub>	1 cc. + 1 cc. diluted 1:5	3	2	1
Mount Sinai anti- <i>coli</i> horse Serum H <sub>60</sub> Bl <sub>188</sub>	Anti-horse rabbit Serum R <sub>358</sub>	0.66 cc. + 0.33 cc.	3	3	0

comm. = commercial.

this table, the expression "positive rabbit" means that there was obtained in the rabbit's prepared skin site a severe hemorrhagic and necrotic lesion 4 to 5 hours after the intravenous injection. The lesions, which were intense in the majority of positive rabbits, were characteristic of the phenomenon of local skin reactivity to bacterial filtrates (6). By "negative rabbit" is meant absence of skin reaction following the intravenous injection.

As is seen from Table II, suspensions of precipitates derived from mixtures of precipitinogen-containing serum with precipitating anti-

serum possess a high reacting potency. The active preparations can be obtained from combinations of some normal animal sera with homologous antisera, as well as from combinations of sera of animals immunized with bacterial antigens with antisera against normal sera of the same animal species.

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" 8, 1931	" "	" "	1:10	3	1	0	2
" 8, 1931	" "	" "	1:50	3	2	0	1
" 8, 1931	" "	" "	1:100	3	3	0	0
June 20, 1931	Normal human serum	" "	Undiluted	3	2	1	0
" 20, 1931	" "	" "	1:5	3	3	0	0
July 23, 1931	" "	" "	Undiluted	3	2	0	1
" 23, 1931	" "	" "	1:5	3	3	0	0
" 29, 1931	" "	Antimeningococcus anti-human horse Serum H <sub>7</sub> Bl <sub>155</sub>	Undiluted	3	0	0	3
" 29, 1931	" "	" "	1:5	3	3	0	0

0.9 to 0.05 cc., respectively. The precipitates obtained in the manner described above gave highly turbid suspensions and had a tendency to settle on the bottom of the test-tube. The aggregates were easily broken up by shaking. The dilutions 1:5 and 1:10 of these suspensions were also quite turbid. In dilution 1:50 the turbidity was slight and in dilution 1:100 the fluid was clear. Various dilutions of the suspensions were tested for reacting potency in rabbits prepared with *B. typhosus* "agar washings" filtrate. The tests were made in the course of the week following the preparation of the suspensions. The results are reported in Table III.

As is seen from Table III, the reacting potency of a given precipitate suspension can be quantitatively measured. As in the experiments with the phenomenon of local skin reactivity to bacterial filtrates, there are also observed natural fluctuations in susceptibility of rabbits employed.

The titrations recorded demonstrate that precipitates derived at various times from mixtures of the same ingredients and proportions may differ in reacting potency.

It is also evident that the amount of precipitate present in a given suspension has no direct bearing on its reacting potency. Thus, a

TABLE IV

*Reacting Potency of Preparations from Completely and Partially Precipitated Mixtures*

Antimeningococcus anti-human horse Serum H <sub>7</sub> Bl <sub>105</sub>	Normal human serum	Amount of precipitate* obtained	Reacting potency of precipitate	Reacting po- tency of super- natant fluid
1 part undiluted	0.1 part undiluted	Medium**	+	+
1 " "	0.1 " diluted 1:2	Maximum	+	0
1 " "	0.1 " " 1:8	Medium	+	+
1 " "	0.1 " " 1:16	Minimum	+	+
1 " "	0.1 " " 1:20	None	0	0

+ = one to three rabbits of each group of three tested showing severe hemorrhagic necrosis. 0 = no reaction obtained in a group of three rabbits.

\* The precipitate was suspended in a volume of 0.25 per cent NaCl solution equal to one-fourth of the volume of horse serum used.

\*\* Prozone.

preparation of only slight turbidity (*i.e.* dilution 1:50 of preparation of May 8, 1931) was potent, whilst several highly turbid preparations (*i.e.* dilutions 1:5 of the remaining suspensions) were totally inactive.

Similar observations were obtained with titrations carried out in the following manner.

Suspensions of precipitates were diluted in 0.85 per cent NaCl solution to a turbidity standard (McFarland nephelometer) of 500 million bacteria per cc. The test dose was 2 cc., per kilo of body weight.

*Preparation I.*—The mixture consisted of one part of antimeningococcus anti-human horse Serum H<sub>7</sub>Bl<sub>105</sub> and one part of human serum diluted 1:4. A suspension of standard turbidity and dilution 1:5 elicited severe reactions in two out of three rabbits tested. Dilution 1:10 gave negative results.

*Preparation II.*—The mixture consisted of one part of antimeningococcus anti-human horse Serum H<sub>7</sub>Bl<sub>27</sub> and one part of human serum diluted 1:4. A suspension of the precipitate of standard turbidity and suspensions two and four times more concentrated gave no reactions in rabbits.

It seems, therefore, that the reacting potency of a serum precipitate is not due to the mechanical effect of an inert colloidal suspension in the blood stream, but to some toxic quality associated with the precipitate. The following experiments were done in order to test this assumption further.

*Reacting Potency of Precipitates and Supernatant Fluids Derived from Completely and Partially Precipitated Mixtures*

In these experiments each preparation was tested in a group of three rabbits. The results are summarized in Table IV.

As is seen from Table IV, mixtures of serum with antiserum in proportions giving maximum precipitation yielded active precipitates and supernatant fluids devoid of reacting potency. Where precipitations were incomplete (*i.e.* in proportions yielding minimum and medium amounts of precipitates) both precipitates and supernatant fluids were potent.

The supernatant fluids were clear at the time of injections; namely, the day following preparation. Those derived from completely precipitated mixtures remained clear for an indefinite length of time. The supernatant fluids derived from partially precipitated mixtures became cloudy after several days in the refrigerator. The latter potent preparations were recentrifuged 2 to 3 times in the course of the following week. When no more cloudiness appeared in the refrigerator they were found on retest totally devoid of reacting potency. It became evident that the potency of supernatant fluids was due to the presence of a precipitate grossly invisible at the time of the injection.

Thus, it can be concluded from the above experiments that the reacting potency of a given preparation does not depend either on the amount of precipitate obtained or on the size of the aggregate formed.

*Reacting Potency of Precipitates and Supernatant Fluids Derived from Mixtures of Bacterial Filtrates with Immune Sera*

When a *B. typhosus* "agar washings" culture filtrate is mixed with an immune antityphoid horse serum in necessary proportions, there



occurs complete neutralization of *B. typhosus* reacting factors (5). The mixtures usually form abundant precipitates. In the experiments reported here, various numbers of reacting factors were mixed with a constant amount of antityphoid horse serum. The mixtures made in the proportions indicated in Table V, precipitates and the supernatant fluids derived from these mixtures were all tested for reacting potency. The results are summarized in Table V.

As is seen from Table V, precipitates derived from mixtures of *B. typhosus* filtrates with homologous immune serum were devoid of

TABLE V

*Reacting Potency of Precipitates and Supernatant Fluids from Mixtures of Bacterial Filtrates with Immune Sera*

Mixtures of <i>B. typhosus</i> reacting factors and antityphoid horse Serum H <sub>4</sub> Bl <sub>22</sub>		Reacting potency of mixtures, per kilo of body weight	Reacting potency of precipitates, per kilo of body weight	Reacting potency of supernatant fluids, per kilo of body weight
Nos. of reacting units	Amount of H <sub>4</sub> Bl <sub>22</sub>			
	cc.			
300	1	0	0	0
400	1	0	0	0
750	1	0	0	0
1000	1	+	+	0
1500	1	+	+	0
2000	1	+	+	0
3000	1	+	+	+

+ = three rabbits tested showing severe hemorrhagic necrosis. 0 = no reactions obtained in a group of three rabbits.

reacting potency if a sufficient amount of serum was used to neutralize *B. typhosus* reacting factors. When an excess of these factors was employed the precipitates proved active. Inasmuch as the potency of the precipitates appeared only with the increase in the amount of *B. typhosus* reacting factors in the mixture, it became evident that these precipitates, in contrast to those derived from serum plus anti-serum mixtures, possessed no independent reacting potency.

Incidentally, it is of interest that in partially neutralized mixtures the non-neutralized *B. typhosus* reacting factors were associated with the precipitates. Thus, the precipitates derived from mixtures of

1000 and more reacting units with 1 cc. of serum were potent, whilst the supernatant fluid derived from a mixture of 2000 reacting units with 1 cc. of serum was inactive.

*Origin of Reacting Potency of Serum Precipitates*

A series of experiments was performed in order to determine which

TABLE VI

*Origin of Reacting Potency of Serum Precipitates*

Group No.	Precipitinogen-containing serum	Precipitating antiserum	Ratio of precipitinogen-containing serum to precipitating serum	Results
1	B. H. antimeningococcus horse Serum 347/169	Anti-horse rabbit Serum R <sub>403</sub>	0.66 cc. + 0.33 cc.	+
2	Mount Sinai antimeningococcus horse Serum H <sub>8</sub> Bl <sub>251</sub>	" "	0.66 " + 0.33 "	0
3	Normal human Serum I	Anti-human rabbit Serum R <sub>392</sub>	1 cc. + 0.2 cc.	0
4	Normal human Serum II	" "	1 " + 0.2 "	0
5	Normal human Serum III	" "	1 " + 0.2 "	0
6	Normal human Serum I	Antimeningococcus anti-human horse Serum H <sub>7</sub> Bl <sub>335</sub>	1 " + 0.2 "	+
7	Normal human Serum II	" "	1 " + 0.2 "	+
8	Normal human Serum III	Antimeningococcus anti-human horse Serum H <sub>7</sub> Bl <sub>403</sub>	1 " + 0.2 "	+

B.H. = New York Board of Health. + = one to three rabbits of each group of three tested showing severe hemorrhagic necrosis. 0 = no reactions obtained in a group of three rabbits.

of the ingredients of serum plus antiserum mixtures was responsible for the reacting potency of the precipitates. The results are reported in Table VI.

As is seen from Table VI, mixture of one horse serum with anti-horse rabbit serum yielded a potent precipitate (Group 1) whilst another

horse serum mixed with the same anti-horse rabbit serum gave an inactive precipitate (Group 2). In these experiments, then, the reacting potency was imparted by the precipitinogen-containing serum. In contrast, several batches of normal human serum mixed with the same anti-human rabbit serum gave inactive precipitates (Groups 3, 4 and 5). However, anti-human antimeningococcus horse serum mixed with the same batches of normal human serum yielded potent precipitates. Here, the reacting potency was, apparently, imparted by the precipitin-containing antiserum. It can be concluded, therefore, that the reacting potency of serum precipitates may originate from either of the ingredients of serum plus antiserum mixtures.

### III

#### *Testing of Inert Colloidal Suspensions for Reacting Potency*

(a) In these experiments each of the substances was tested in a group of six prepared rabbits. The dose was 1 cc., per kilo of body weight. The suspensions made in 0.85 per cent NaCl solution were as follows: 4 per cent charcoal, 2 per cent infusorial earth, 4 per cent Witte's peptone, 4 per cent silicic acid and 10 per cent gelatine. All the substances were devoid of reacting potency. According to Sickles (7) galactose, gelatine and India ink have no reacting potency.

(b) Sickles reported (7) that agar was able to elicit reactions in rabbits previously prepared with meningococcus toxin. These experiments were repeated in the following manner.

The agar was dissolved in 0.85 per cent NaCl solution and sterilized by autoclaving at 250°F. for 15 minutes.

Rabbits were prepared with *B. typhosus* "agar washings" filtrate, as in above experiments. The intravenous dose was 2 cc., per kilo of body weight, of agar suspension tested. The suspensions contained 0.8, 0.5, 0.3, 0.2, 0.15, 0.08 and 0.01 per cent of agar. The dose of 2 cc. of 0.2 per cent of agar was the smallest quantity which elicited reactions in about 50 per cent of rabbits tested. Smaller doses (*i.e.*, 0.08 and 0.05 per cent) gave doubtful reactions. No reactions were obtained with 0.01 per cent of agar.

Sickles (7) showed that agar had no skin-preparatory effect. This has been also corroborated.

## DISCUSSION AND CONCLUSIONS

Early in the work it was noticed that the skin-preparatory potency of various bacterial filtrates did not parallel their reacting potency. Thus, the majority of *B. typhosus* culture filtrates contained between 40 to 60 skin-preparatory units (2) and between 500 to 700 reacting units per 1 cc. (5). On the other hand, some meningococcus culture filtrates contained only ten preparatory units and between 2000 to 3000 reacting units. Moreover, culture filtrates of some bacterial species (*i.e.* streptococcus) (8), agar (7) and serum precipitates, as shown in this paper, were found lacking skin-preparatory effect but possessing considerable reacting potency. In the latter instances, the phenomenon could be elicited when rabbits were prepared with some bacterial filtrate of high skin-preparatory potency. Gratia and Linz reported that vaccine virus was able to elicit a state of reactivity to *B. coli* culture filtrates injected intravenously (9). According to Klein (10) vaccine virus had no reacting potency for skin areas prepared with potent bacterial filtrates.

It appears from observations made thus far that skin-preparatory and reacting factors are substances independent of each other. For this reason, the elicitation of the state of reactivity and production of injury in the reactive tissue can be considered as two independent phases of the phenomenon under discussion.

The first phase, *i.e.* elicitation of the state of reactivity, was interpreted by the present author as a functional disturbance in the tissue cells bringing about a transient state of vulnerability. The state is characteristically induced by bacterial filtrates<sup>2</sup> or by infection and is not a mere manifestation of the process of inflammation according to our present understanding of it (1).

The data presented in this paper concern the second phase of the phenomenon; *i.e.*, production of injury *via* the blood stream in tissue of induced vulnerability.

As has been seen, precipitates derived from mixtures of serum precipitinogen with precipitating antiserum were able to elicit severe

<sup>2</sup> Debonera, Tzortzakos and Falchetti (11) claimed that paraffin oil also induced a state of reactivity. Their observation could not be corroborated in this laboratory.

injury in skin sites prepared with bacterial filtrates. The amount of precipitate present in a given suspension had no direct bearing on its reacting potency. Some abundant serum precipitates as well as precipitates derived from neutralized mixtures of *B. typhosus* culture filtrates with immune sera were found inactive. The reacting potency did not depend on the size of the aggregate formed inasmuch as clear supernatant fluids obtained by centrifugalization of partially precipitated serum plus antiserum mixtures were able to elicit severe reactions. With the exception of agar, inert colloidal suspensions thus far tested (*i.e.* charcoal, infusorial earth, peptone, silicic acid, gelatine, galactose and India ink) possessed no reacting potency. The evidence thus far accumulated, therefore, points to the fact that the reacting potency of serum precipitates is not due to the mechanical effect of colloidal particles in the blood stream but to some toxic factors liberated or formed in the serum through the colloidal disturbance induced by the process of precipitation.

As pointed out before (1) the *sine qua non* of the phenomenon of local skin reactivity to bacterial filtrates is that the second injection be given *via* the blood stream. It is conceivable that, as in the case of sera, bacterial filtrates have no direct reacting potency but that they induce a colloidal disturbance in the blood which brings about liberation or formation of reacting factors. It remains to determine whether the effect of agar is due to a similar mechanism.

The phenomena of local skin reactivity to bacterial filtrates and serum precipitates seem to be best defined at present as the production of injury in tissues during a transient state of vulnerability induced by bacterial filtrates. The injury is produced by means of toxic factors formed in the blood stream through a colloidal disturbance in the latter.

In connection with the above described formation of reacting factors through a colloidal disturbance in blood serum, observations of Rous and his coworkers made in 1919 and 1920 are highly interesting. In order to remove the shock-producing property of a precipitating serum, they attempted repeated absorptions of hemolysins, hemagglutinins and precipitins. The absorbed serum, however, was able to engender shock. On the basis of these observations, they concluded that "there remains the interesting possibility of the presence in the serum

of a hitherto unrecognized toxic antibody. Further work alone can justify any speculation in this direction" (12).

As reported in this paper, sera concentrated by chemical means contained a considerable amount of toxic substances which apparently originated from contaminants introduced during the process of concentration. It seems worth while to investigate whether the substances bear any relation to primary toxic effects and chills observed in human beings under treatment with concentrated immune sera.

#### SUMMARY

Observations reported here seem to demonstrate the liberation or formation of a toxic principle in blood serum through a colloidal disturbance in the latter. The principle is able to elicit severe injury in tissues made vulnerable by a bacterial filtrate.

The author wishes to express his gratitude to Miss Edith Mandel for her capable technical assistance.

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# EXPERIMENTS ON THE PURIFICATION AND CONCENTRATION OF THE VIRUS OF POLIOMYELITIS\*

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For a thorough understanding of the nature and mode of action of filterable viruses, it is necessary that they be separated from the tissue substances with which they are associated. Moreover, the methods whereby such purification may be accomplished, are in themselves capable of yielding additional data concerning the properties of the virus in question. The purpose of this communication is to describe procedures effective in separating the virus of poliomyelitis from most of its associated tissue constituents and in its subsequent concentration.

The methods follow closely those which enabled Willstätter and his coworkers to isolate enzymes in their purest known form. Numerous investigators (1) have reported the capacity of various colloidal suspensions to adsorb filterable viruses. In most instances the viruses appear to be inactivated in the adsorbed state, and in the case of poliomyelitis virus, Amoss (2) states that "the presence of colloidal substances with adsorptive power destroys the virulence after 1 or 2 days." Gilde-meister and Herzberg (3) adsorbed bacteriophage on kieselguhr and succeeded in subsequently eluting it with dilute ammonia. Their results were confirmed by Callow (4) and Kligler and Olitzki (5). The latter investigators (5) obtained similar results with fowl-pox virus, using kaolin as the adsorbing agent. Recently, Rhoads (6) described the adsorption and inactivation of poliomyelitis virus on aluminum hydroxide Type C of the Willstätter series, and showed that adsorption occurred at pH 5.5 and 7.0, but not at pH 8.8. It appeared, therefore, that if the inactivation of the virus is not irreversible, it should be possible to adsorb it at an acid pH, and elute at an alkaline pH. That the inactivation is reversible and that elution of the virus is possible, has already been reported in a preliminary communication (7).

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## EXPERIMENTAL

*Poliomyelitis Virus.*—The virus used in the following experiments is the “mixed Rockefeller Institute strain” which has undergone numerous monkey passages in the Research Laboratories of the New York City Department of Health.

*Preparation of Aluminum Hydroxide Gel.*—Alumina gel C was prepared essentially according to the method of Willstätter and Kraut (8), excepting that the centrifuge was used instead of natural sedimentation. An effective gel was thus obtained, the process requiring only 2 days as compared with 2 weeks or more in the original procedure. Distilled water must be used throughout; in the preparation of a large quantity of this gel, tap water was used once and a product of entirely different physical and adsorptive properties was obtained. The following is the procedure for preparing 250 cc. of the gel.

To distilled water at 70°C., sufficient concentrated ammonia is added to make a 4 per cent solution. A filtered solution of 50 gm.  $\text{Al}_2(\text{SO}_4)_3 \cdot 18\text{H}_2\text{O}$  in 150 cc. of water at 65°C. is rapidly poured into the ammonia, and the mixture shaken vigorously for 15 minutes. The gel is then centrifuged until a well packed sediment is obtained; the supernatant water-clear liquid is poured off. The sediment is made up to 1000 cc. with water, shaken, and recentrifuged; this process is repeated five times. After the fifth decantation, 400 cc. of 4 per cent ammonia at 70°C. is added to the sediment and the mixture shaken for 15 minutes. It is then centrifuged, the supernatant liquid poured off, and the sediment made up to 1000 cc. with water. After shaking, it is again centrifuged. The supernatant fluid is water-clear until about the ninth or tenth washing, when it becomes opalescent. When this stage is reached the gel is washed once more and after centrifugation and decantation, the sediment is made up to 250 cc. with water and thoroughly shaken with glass beads. The gel is standardized by determining the quantity of  $\text{Al}_2\text{O}_3$  per cc., which is accomplished by drying 5 cc. in a crucible at 110°C., igniting, and weighing the residue. When the quantities given above are used, different preparations contain from 21–25 mg.  $\text{Al}_2\text{O}_3$  per cc.

*The Elution of Poliomyelitis Virus*

The purpose of this experiment was to determine whether or not the absorption of virus was complete or nearly complete, and whether the virus can then be removed from the gel in an active state. A highly centrifuged monkey cord suspension has an opalescent supernatant liquid, the particles of which are carried down with the alumina gel sediment; to eliminate the possibility of extracting virus from such tissue particles during the eluting process, a Seitz filtrate was used in this experiment.

*Procedure.*—To 5 cc. of alumina gel C, 1 cc. of  $\text{M}/15 \text{KH}_2\text{PO}_4$  and 5 cc. of a Seitz filtrate of a 5 per cent poliomyelitis monkey cord suspension were added.

Immediate flocculation of the gel occurred. The mixture was shaken for 20-30 minutes and left in the refrigerator for 3-4 hours. It was then shaken again and centrifuged for 20 minutes or until the densest possible packing of the gel occurred. The supernatant liquid which had a white (lipoid-containing (?)) cake at its surface was poured off. The sides of the tube and the surface of the gel were carefully washed with distilled water. The sediment was now mixed with 5 cc. of  $M/15 Na_2HPO_4$ . The gel which was formerly flocculated became homogeneous; after shaking for 20 minutes it was left at room temperature overnight. The following morning it was again shaken and then centrifuged. The supernatant liquid was colorless and water-clear without any cake at its surface. (It is interesting to note that there were other preparations of alumina gel C and of virus which failed to show the above mentioned flocculation reaction or separation of lipoid-containing (?) material, and nevertheless worked equally well as regards adsorption and elution.) The original virus filtrate, the adsorbed supernatant liquid, and the eluate were then diluted to the same volume. 1 cc. of the dilutions (equivalent to 0.05 cc. of the original) was injected intracranially into monkeys.

TABLE I

*Adsorption and Elution of Poliomyelitis Virus*

Substance tested	Dose	Monkey No.	Result
	cc.		
5 per cent Virus Filtrate 593 . . . .	0.05	1	Typical poliomyelitis—6 days
Adsorbed supernatant liquid . . . .	0.05	2	No poliomyelitis
$M/15 Na_2HPO_4$ eluate . . . . .	0.05	3	Typical poliomyelitis—5 days
5 per cent Virus Filtrate 590 . . . .	0.05	4	Typical poliomyelitis—6 days
Adsorbed supernatant liquid . . . .	0.05	5	No poliomyelitis
$M/150 Na_2HPO_4$ eluate . . . . .	0.05	6	Typical poliomyelitis—10 days

In Table I it will be seen that with the doses used the adsorption of the virus is probably complete or nearly complete, and that the  $M/15 Na_2HPO_4$  eluate, volumetrically equivalent to the original virus filtrate, produced symptoms somewhat more rapidly even than the control. Chart 1 shows the strikingly similar course of the temperature and paralysis in Monkey 1, injected with the original virus, and Monkey 3, injected with the  $M/15 Na_2HPO_4$  eluate.

In another experiment the effectiveness of  $M/150 Na_2HPO_4$  as an eluting agent was determined. The previous procedure of adsorption and elution was exactly duplicated. Table I shows that the  $M/150$

$\text{Na}_2\text{HPO}_4$  eluate also produced typical poliomyelitis, although the incubation period was twice as long as with the  $\text{M}/15 \text{Na}_2\text{HPO}_4$  eluate.

*Selective Washing of the Alumina Gel C-Virus Complex*

The purpose of this experiment was to determine whether or not it would be possible to wash away selectively any inactive organic substances from the sediment containing the adsorbed virus. Since the virus is adsorbed at an acid pH, it appeared theoretically possible to extract soluble substances which were carried down in the sediment

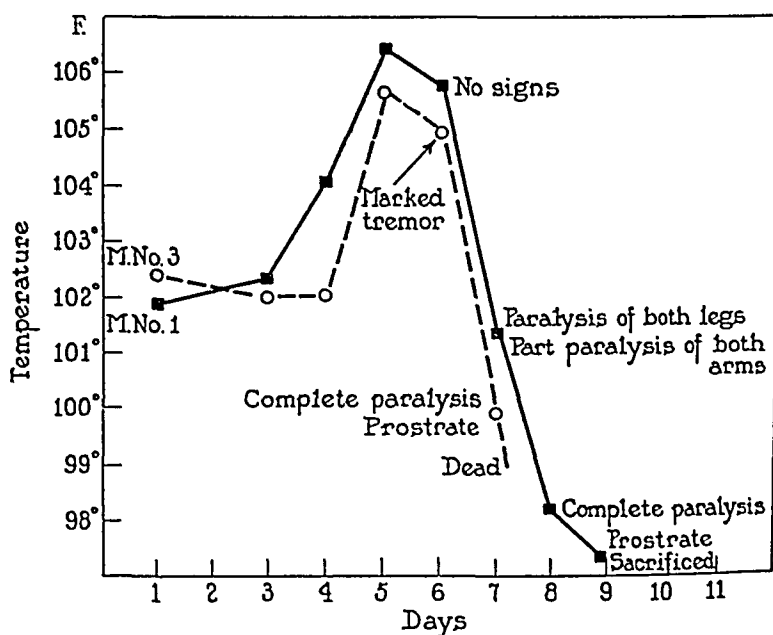


CHART 1. Course of temperature and paralysis in monkeys injected with original and eluted virus.

■——■ Monkey 1, original virus.  
○-----○ Monkey 3, eluted virus.

mechanically and by imbibition with a salt solution containing  $\text{M}/15 \text{KH}_2\text{PO}_4$ .

*Procedure.*—To 5 cc. of a 5 per cent Seitz-filtered pooled virus preparation, 5 cc. alumina gel C and 1 cc.  $\text{M}/15 \text{KH}_2\text{PO}_4$  were added. This mixture was shaken and centrifuged as previously described. The supernatant liquid was poured off and 9 cc.  $\text{M}/5 \text{NaCl}$  and 1 cc.  $\text{M}/15 \text{KH}_2\text{PO}_4$  were added to the sediment. The sediment was stirred and the mixture shaken vigorously for 20 minutes, allowed to

stand 4 hours, and then centrifuged until the sediment was packed as densely as possible. The supernatant liquid was poured off and 5 cc. of  $m/15$   $Na_2PO_4$  were added for elution. The original Seitz-filtered virus, the adsorbed supernatant liquid, the  $m/5$   $NaCl$ - $m/15$   $KH_2PO_4$  washings, and the eluate were tested for virus content by intracranial injection into monkeys.

The results, shown in Table II, indicate that the  $m/5$   $NaCl$ - $m/15$   $KH_2PO_4$  washings, like the adsorbed supernatant liquid, contain practically no virus, whereas the  $m/15$   $Na_2HPO_4$  eluate is probably almost as potent as the original virus preparation. Analysis of the  $NaCl$ - $KH_2PO_4$  washings showed the presence of nitrogen-containing organic substances though quantitatively less than that found in the  $m/15$   $Na_2HPO_4$  eluate.

TABLE II

*Effect of Washing Alumina Gel C-Virus Complex with  $m/5$   $NaCl$ - $m/15$   $KH_2PO_4$*

Substance tested	Dose	Monkey No.	Result
	cc.		
5 per cent virus filtrate. ....	0.01	7	Typical poliomyelitis, 7 days
Adsorbed supernatant liquid. ....	0.10	8	No poliomyelitis
$NaCl$ - $KH_2PO_4$ washings. ....	0.50	9	No poliomyelitis
$m/15$ $Na_2HPO_4$ eluate. ....	0.01	10	Typical poliomyelitis, 7 days

### *Concentration of Poliomyelitis Virus*

Certain immunological and chemical studies on filterable viruses are either impossible or unreliable with the concentrations obtained by the ordinary methods of extraction. To obtain a concentrated solution of poliomyelitis virus two procedures were considered: (1) concentration by elution in a volume smaller than that of the original virus preparation, (2) concentration by dialysis and distillation *in vacuo*.

*Procedure.*—105 gm. of poliomyelitis monkey cords were ground with sand and saline (2100 cc.), shaken for an hour, and allowed to stand in the refrigerator overnight. After centrifugation the supernatant liquid was filtered through paper pulp and then through a large Seitz filter under positive pressure. Adsorption was carried out as follows: 1000 cc. of alumina gel C (24 mg.  $Al_2O_3$  per cc.) plus 200 cc.  $m/15$   $KH_2PO_4$  plus 2000 cc. of the virus filtrate. (The virus filtrate was sterile, but the phosphate and alumina gel were added unsterilized and without

preservative.<sup>1</sup>) The mixture was shaken for an hour and allowed to stand in the refrigerator overnight. It was then centrifuged until the densest possible packing of the sediment had occurred. The supernatant liquid, which was water-clear and had no lipid cake at the surface, was poured off. The sediment was taken up in a mixture of 1800 cc. M/5 NaCl and 200 cc. M/15  $\text{KH}_2\text{PO}_4$ . After stirring, it was shaken for an hour and allowed to stand for 4 hours. This was centrifuged and the washing was repeated with another 2000 cc. of NaCl- $\text{KH}_2\text{PO}_4$  solution. The sediment from the second washing was eluted with 1000 cc. of M/15  $\text{Na}_2\text{HPO}_4$  (Eluate 1). The sediment from the first eluate was then treated with another 1000 cc. of M/15  $\text{Na}_2\text{HPO}_4$  (Eluate 2). The original virus preparation, the NaCl- $\text{KH}_2\text{PO}_4$  washings, the adsorbed supernatant liquid, and both eluates were tested for virus content by intracranial injection into monkeys.

The data presented in Table III show (1) that the minimal effective dose in the original virus filtrate is contained in 0.005 cc., < 0.001 cc., (2) that fairly complete adsorption occurred, (3) that washing with M/5 NaCl-M/15  $\text{KH}_2\text{PO}_4$  removed very little or practically no virus, (4) that one elution with half the original volume leaves a large amount of virus on the alumina gel, which can be recovered by further elution. It appeared from these results that concentration by elution with diminished volumes would be wasteful of large amounts of virus and therefore impracticable.

Before attempting to distill virus-containing solutions *in vacuo*, it was necessary, of course, to be rid of the relatively large amounts of salts present in them. No data were found in the literature regarding dialysis of poliomyelitis virus. Callow (4) in her study of a staphylococcus bacteriophage found that it diffused readily through collodion membranes made with an alcohol concentration higher than 60 per cent, whereas sacs made with 50 per cent alcohol-50 per cent ether were always impermeable. In the following experiment; collodion was used which contained 24 per cent alcohol and 76 per cent ether. Bags were prepared by coating large flasks with the collodion, repeating process three times to give stronger sacs as well as to diminish the chances of imperfections.

The first and second eluates, previously described and tested (Table III), were combined and 1500 cc. were dialyzed against running tap water for 48 hours, at

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<sup>1</sup>It now appears advisable to work either with sterile solutions under aseptic conditions or with suitable preservatives.

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the end of which time the solution inside the bag no longer gave a test for phosphate; chloroform was added to the dialyzing solution to prevent gross contamination. The dialyzed solution was distilled under diminished pressure (6-7 mm. of mercury) at an outside temperature which did not exceed 42°C. and an inside temperature which did not exceed 30°C. The small distillation flask, which was immersed in the water bath, never contained more than 30-40 cc., the solution

TABLE III  
*Effect of the Volume of Eluting Fluid on the Amount of Virus Recovered*

Substance tested	Dose cc.	Monkey No.	Result
Original pooled virus filtrate— 5 per cent	0.01	11	No poliomyelitis*
	0.005	12	Typical paralysis, 7 days
	0.001	13	No poliomyelitis
	0.0005	14	No poliomyelitis
Adsorbed supernatant liquid m/5 NaCl-m/15 KH <sub>2</sub> PO <sub>4</sub> wash- ings	0.1	15	No poliomyelitis
	0.2	16	No poliomyelitis
First m/15 Na <sub>2</sub> HPO <sub>4</sub> eluate (one-half volume of the origi- nal virus filtrate)	0.01	17	Typical paralysis, 7 days
	0.005	18	Typical paralysis, 10 days
	0.001	19	No poliomyelitis
Second m/15 Na <sub>2</sub> HPO <sub>4</sub> eluate (one-half volume of the origi- nal virus filtrate)	0.005	20	Typical paralysis, 7 days

\* Monkeys 11 and 12 were both injected with solution from the same tube; yet No. 11 which received twice as much as No. 12 did not show any sign of poliomyelitis. That Monkey 11 was not entirely refractory is evident from the fact that it developed typical poliomyelitis when injected with a large dose of virus 2 months later. This appears to be a very good example of the relative resistance of some monkeys to the disease.

being delivered into it from a separate flask at approximately the same rate as the water distilled off. Towards the end of the distillation foaming became rather marked, necessitating the addition of a few drops of octyl alcohol. The 1500 cc. of solution were reduced to a volume of 20 cc.; a relatively large amount of insoluble matter, both amorphous and crystalline settled out. After centrifugation, the supernatant liquid was dialyzed against 20 liters of distilled water for 24 hours, during which time the volume increased to about 100 cc. A second distillation

*in vacuo* was performed to reduce the volume of the solution to 30 cc. After centrifugation, 10 cc. of the concentrated supernatant liquid were adsorbed on 10 cc. of alumina gel C, and subsequently eluted in 10 cc. M/15  $\text{Na}_2\text{HPO}_4$ .

The original concentrated solution and its eluate were tested for virus content by intracranial injection into monkeys, the results being recorded in Table IV. The potency of the original, unconcentrated Seitz-filtered virus preparation was such that 0.005 cc., < 0.001 cc. of it (Table III) was necessary for producing poliomyelitis in a monkey. The results in Table IV indicate that the minimal effective dose of virus in the concentrated preparation was 0.0005 cc., < 0.0001 cc., and that

TABLE IV  
*Potency of Concentrated and Purified Virus Preparation*

Substance tested	Dose cc.	Monkey No.	Result
Virus eluates dialyzed and concentrated 50 times by volume	0.0010	21	Typical paralysis, 8 days
	0.0005	22	Typical paralysis, 11 days
	0.0001	23	No paralysis
M/15 $\text{Na}_2\text{HPO}_4$ eluate of concentrated virus preparation	0.0003	24	Typical poliomyelitis, 18 days
	0.0001	25	No paralysis

another adsorption and elution on this concentrated virus yielded a product which was potent in 0.0003 cc., < 0.0001 cc. Thus with a concentration of 50 times by volume, the increase in potency was only about tenfold. With the data at hand, it appears almost impossible to account for the manner in which this amount of virus was lost. If the virus had diffused readily through the dialyzing membranes, none or extremely little should have been left within the sacs after the prolonged dialysis against running tap water. Nevertheless, it appeared interesting to determine whether small amounts of virus could possibly diffuse through these rather thick and relatively impermeable collodion membranes. To do this 5 cc. of the combined unconcentrated eluates were dialyzed in a tested collodion bag (prepared as previously described) against 5 cc. of distilled water for 48 hours. At the end of that time, 2 cc. of the water outside the bag were injected intracranially into a monkey. This monkey came down with

typical poliomyelitis in 17 days. Considering the amount that was injected, and the delayed incubation period, the quantity of virus that had passed through the membrane could not have been very large. No attempt will be made here to discuss the possible significance of this fact on the nature of the virus, but it suffices to say that it may perhaps account for part of the loss during the process of concentration. It is also possible that part of the virus may have been lost with the solid matter that settled out during the distillation. However, there are probably many other factors to account for the diminished yield.

Nevertheless, the results of this experiment indicate that it is possible by repeated adsorptions, selective washings, and elutions, and by distillation *in vacuo* to obtain a purer and more concentrated preparation of poliomyelitis virus. What is, of course, of the greatest interest is that one can apply the methods used in the purification of non-living chemical substances to the isolation of the virus of poliomyelitis.

#### *Chemical and Immunological Tests on Original and Eluted Virus*

Many of the original Seitz filtrates of 5 per cent poliomyelitis monkey cord suspensions had no coagulable substances on heating at neutrality or with dilute acetic acid, and gave negative biuret and ninhydrin reactions. These tests could not be used therefore as criteria for the presence or absence of neuroproteins in the eluted virus. Determinations of the total solids and the inorganic residue upon ignition revealed that after one adsorption, the eluate contains probably less than 10 per cent of the organic constituents contained in the original virus filtrate. The serum of horses treated over a long period with poliomyelitic monkey cords and brains contains some precipitins for normal monkey neuroproteins, although in very low titer only. It has been observed, however, that definite flocculation occurred only with coarse suspensions of monkey brain and cord, and even with slightly opalescent solutions obtained after prolonged centrifugation, but none at all or at the most a very indefinite reaction with the Seitz or Berkefeld filtrates of these same suspensions. For this reason the precipitin reaction also appeared to be unsatisfactory as a criterion for the presence or absence of small amounts of neuroprotein in the purified, water-clear solutions. Injections of monkey brain and cord suspensions into rabbits over a period of a month with suitable rest



intervals failed to produce a serum which would precipitate Seitz or Berkefeld filtrates of neuroprotein. Finally, guinea pig sensitization was resorted to as a method for testing small amounts of monkey neuroprotein. Twelve guinea pigs were injected intraperitoneally with 2 cc. each of a highly centrifuged 10 per cent normal monkey cord and brain emulsion, and twenty guinea pigs with 2 cc. each of the whole, uncentrifuged emulsion. Half the guinea pigs were tested for anaphylaxis after 21 days and the other half after 30 days, 1 cc. of the 10 per cent Seitz filtrate intravenously being the shocking dose. In not a single instance was there definite and typical anaphylactic shock. There were a few instances of slight and transitory dyspnea, reactions, which, however, were sufficiently indefinite not to be considered positive. It appeared therefore that either monkey brain and cord emulsions were poor antigens or no antigens at all as concerns the guinea pig. There was, therefore, no way of showing whether or not the purified preparations of virus were associated with small amounts of monkey neuroproteins.

However, it is interesting to note that the nitrogen content<sup>2</sup> per cc. of the original (5 per cent) unconcentrated and unpurified virus preparation (minimal effective dose—0.005, < 0.001 cc.) was 0.188 mg., whereas that of the last eluate of the concentrated preparation (minimal effective dose—0.0003, < 0.0001 cc.) was 0.04 mg. Thus with an approximate tenfold increase in potency there was an almost fivefold decrease in nitrogen content. This final purified and concentrated preparation failed to give the biuret, ninhydrin, and xanthoproteic reactions.

It is evident, of course, that the concentrated and purified preparation obtained in the last experiment does not represent pure virus. However, it does appear possible that by the use of suitable antiseptics throughout the various procedures, numerous repeated adsorptions, selective washings, and elutions, with concentration by distillation *in vacuo*, may yield concentrated and highly purified virus preparations.

#### SUMMARY AND CONCLUSIONS

Methods employed by Willstätter and his coworkers in the isolation and purification of enzymes have been applied to the virus of polio-

<sup>2</sup> The nitrogen content was determined by micro Kjeldahl through the courtesy of Dr. H. Sobotka and Miriam Reiner of the Chemical Laboratory of The Mount Sinai Hospital.

myelitis. Rhoads (6) showed that alumina gel C mixed with poliomyelitis virus in certain proportions at an acid pH resulted in the adsorption and inactivation of the virus. The experiments in this communication confirm Rhoads' observation, and show further that the adsorption as well as the inactivation are reversible; *i.e.*, by changing the pH to the alkaline side with  $M/15 \text{ Na}_2\text{HPO}_4$  it is possible to free the virus in a state in which it is again capable of producing typical poliomyelitis. These experiments show also that by this process the virus undergoes considerable purification by diminution in the concentration of non-virus-containing substances. Washing the alumina gel C-virus complex with  $M/5 \text{ NaCl-M/15 KH}_2\text{PO}_4$  apparently dissociates no virus but is capable of freeing a certain amount of extractible organic substances. Furthermore, it is possible to increase the degree of purity and concentration of the virus by distillation *in vacuo*, and subsequent repeated adsorptions and elutions. By such partial purification and concentration, a virus solution with a minimal effective dose (as regards the production of typical poliomyelitis) of 0.0003 cc. was obtained. This solution had 0.04 mg. N per cc., and gave negative biuret, xanthoproteic, and ninhydrin reactions.

These methods offer an opportunity for the preparation of a quantity of sufficiently purified and concentrated poliomyelitis virus to warrant the beginning of a study of its chemical nature; they also offer a means of anchoring the virus to an insoluble, and centrifugable substance (alumina gel C), which promises to facilitate various immunological studies, that might otherwise have been impossible.

I wish to thank Professor William H. Park for the stimulating interest he has taken in this work.

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# THE DETECTION OF POLIOMYELITIS VIRUS IN SO CALLED ABORTIVE TYPES OF THE DISEASE

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## PLATE 19

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In another publication dealing with certain clinical and epidemiological aspects of poliomyelitis (1), we have discussed the rôle which so called abortive cases may play in the spread of this disease and have pointed out that discussions dealing with so called abortive poliomyelitis demand a definition of this rather loose term. Definitive statements are necessary because there is little unanimity of opinion as to what constitutes abortive poliomyelitis, although popular and practical usage today tends to designate those cases as abortive, in which signs pointing to meningitic or myelitic involvement exist, but in which no paralysis develops. In other words this concept rests upon the tenet that one cannot diagnose clinical poliomyelitis in the absence of signs pointing to meningitic or myelitic involvement. This is quite different from the view originally proposed by Wickman (2). He felt that the abortive types of poliomyelitis might be manifest by such minor symptoms as fever of short duration, sore throat, headache, and vomiting, and that these symptoms might be the only manifestations of the disease. In his experience the abortive types were one-third as frequent as the frank cases, but he quoted certain estimates, made during epidemics, in which abortive cases outnumbered the frank cases. Draper reflects this same concept intimating that the first phase of the "dromedary" form of clinical poliomyelitis which is often characterized merely by headache, fever, sore throat, and listlessness, may be the only recognizable phase of the disease in 50 to 80 per cent of all cases of poliomyelitis (3). The obvious difficulty of subjecting this situation to analysis is that such nondescript symptoms as those just mentioned do not furnish a clinical picture

which can be readily identified, and probably this is the reason why most physicians are unwilling to make a diagnosis of either poliomyelitis, or abortive poliomyelitis, in the absence of tangible specific signs; namely, signs pointing to meningitic or myelitic involvement. In any event the question of uncertainty has excluded such cases from statistics on poliomyelitis (4, 5), although it is needless to point out, that, if the disease is thus limited, such a concept will enormously influence views not only on its epidemiology but also on its fundamental nature.<sup>1</sup> Unfortunately the problem of definition of abortive poliomyelitis is far from settled, and the uncertainty which has clouded Wickman's original contentions, and Draper's subsequent concepts is that the clinical entities they embrace are vague, and their relation to poliomyelitis is based essentially on circumstantial evidence. Furthermore, although many have suspected that it is through the abortive cases that the mass immunization of most adult populations to poliomyelitis occurs, this contention has apparently resisted recent experimental proof (6).

In our own studies on these problems (1), which were made in New Haven during the summer of 1931, we were immediately confronted with the numerical importance of possible mild and fleeting attacks of the disease and the problems which they offered in the field of practical clinical medicine and epidemiology. It seemed to us that common usage of the term abortive poliomyelitis had in the past proved so ambiguous, that, to define the issues in this disease, we would employ instead the term *characteristic minor illness*, in association with poliomyelitis, so that ground might be cleared for a de-

<sup>1</sup> Almost 20 years ago Frost discussed this question in the following terms.

"The inclusion of definite abortive cases more than doubles the secondary attack rate. The inclusion of suspected abortive cases more than quadruples it, giving to poliomyelitis an apparent contagiousness comparable to that of scarlet fever and diphtheria, diseases which are generally considered highly contagious. It is evident, therefore, as stated before, that the question of contagiousness of poliomyelitis as determined by epidemiologic studies, hinges largely upon the question of which are and which are not cases of poliomyelitis. To omit from epidemiologic studies cases without paralysis, to use a somewhat exaggerated simile, is like undertaking to study the epidemiology of typhoid fever by considering only cases of hemorrhage. The inclusion of cases without paralysis is, on the other hand, open to the very reasonable objection that the epidemiology of the disease may thereby be confused by the consideration of cases in no way related to the disease in question" (5).

finite study. Essentially these *characteristic minor illnesses* owe their identity merely to the fact that they appear during an epidemic of poliomyelitis. We have reviewed the clinical symptomatology of a series of 136 examples of these illnesses in another publication (1). This analysis revealed little whereby they might be identified clinically, although, as Draper has pointed out, there is a close similarity between their symptomatology and that of the first phase of the "dromedary" form of clinical poliomyelitis. Our estimates (1) of their relative frequency showed that (a) in a large series of families these characteristic minor illnesses developed in about 40 per cent of children under 5 with familial exposure to poliomyelitis, as opposed to a 4 per cent incidence of secondary familial cases of frank poliomyelitis in the same group of families; or, in other words, in this selected group the former were about ten times as frequent as the latter; and (b) in each of three communities in which estimates were made during the epidemic period, these examples of minor illness were six times as frequent as the frank cases of poliomyelitis. However, the most significant observation of this study was the detection of the virus of poliomyelitis in the nasopharynx of two children suffering from these characteristic minor illnesses and in the present communication details of its detection and isolation will be given. We believe that this finding furnishes more than circumstantial evidence that there is a common causal relationship between frank poliomyelitis and illnesses corresponding to Wickman's abortive types.

In order that the experiments to be described may be compared with previous studies dealing with the isolation of the virus of poliomyelitis from the human nasopharynx, the literature on this subject will first be reviewed.

#### *Review of the Literature on the Isolation of the Virus from the Throat*

(a) *Frank Cases.*—Shortly after the earliest successful experiments upon the transmission of poliomyelitis to monkeys and with the development of strains of the virus which were highly potent for monkeys, Flexner and Lewis (7) succeeded in 1910 in isolating the virus from Berkefeld filtrates of extracts of the nasal mucous membrane excised from monkeys suffering with the experimental disease. The corollary to this experiment or the isolation of the virus from human tonsillar tissue and pharyngeal mucosa obtained from a fatal case of poliomyelitis, was soon supplied by Landsteiner, Levaditi, and Pastia (8). At about the same time

Flexner and Clark (9) reported several similar human isolation experiments of this type, drawing attention to the fact that when they injected filtrates of the tonsillar and nasal tissues, they were unable to recover the virus; but when the same unfiltered material was rendered bacteria-free with 0.5 per cent phenol, the virus could be recovered. Subsequently it has become a well established fact that poliomyelitis virus may be detected in washings from the throats of acute cases of frank poliomyelitis, but rarely from human throats under other conditions.<sup>2</sup> Lucas and Osgood (11) have isolated it from the throat of a child, 4 months after the acute stage of a second attack of poliomyelitis, but as far as we know this is the only example of the human convalescent carrier in this disease.

(b) *Healthy Carriers*.—To our knowledge there are but two examples of the detection of the virus in nasopharyngeal washings taken from healthy familial contacts (12, 13). In the observation of this type reported by Flexner, Clark, and Fraser (12) definite success was obtained in one instance, which will be described in some detail.

Pooled saline washings (about 150 cc.) were obtained from both parents of a child who had recently had poliomyelitis. These washings were taken on the 16th day after the onset of their child's case. The fluid was shaken and passed through a Berkefeld filter and 1.5 cc. was injected the same day into the sheath of each sciatic nerve and 140 cc. into the peritoneal cavity of a monkey who subsequently developed poliomyelitis. The virus thus isolated was passed to subsequent monkeys. The authors concluded that the parents of a known case of poliomyelitis, neither of whom showed any symptoms of illness, harbored the virus in the nasopharynx.

A year later Kling and Pettersson (13) reported a single instance of the successful isolation of the virus from pooled washings taken from four healthy members of a family in which one member had recently died of poliomyelitis. The washings were obtained on the 5th day from the onset of the fatal case and were injected into a monkey intracerebrally and intraperitoneally 15 days later. The investigators used large quantities of washings amounting to 1 liter of fluid, which they subsequently filtered through a Berkefeld candle after concentrating the material to 75 cc. by vacuum distillation at 35–38°C. The experimental disease was induced in the monkey inoculated with this material and the virus was successfully passed to a second monkey.

Knowledge concerning the healthy human carrier of the virus of poliomyelitis rests largely upon the results of these two observations both of which were obtained with pooled washings. The number of similar attempts to isolate the virus

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<sup>2</sup> We have recently gone over the well known work of Kling, Pettersson, and Wernstedt (10), who reported the detection of poliomyelitis virus from a rather high percentage of examples of minor illnesses and from healthy contacts. The criteria which these authors used for the detection of the virus are so different from those in use today that it is difficult to evaluate their results.

from healthy contacts is not known, but it is probable that failures greatly outnumber positive results. It is evident, of course, that the significance of our own attempts (subsequently described in this paper) to demonstrate the virus in minor illnesses contemporary with familial or community cases of poliomyelitis could be best evaluated if we knew the healthy carrier rate, but unfortunately this information is still lacking.

(c) *Abortive Cases.*—The literature affords few examples of the isolation of the virus of poliomyelitis from so called abortive cases. The claims of the Swedish investigators (10) were that from seven examples of minor illnesses occurring in association with poliomyelitis, the virus was isolated in two instances, but a careful analysis of their results would lead us to believe that a single questionably positive result was obtained from this group.

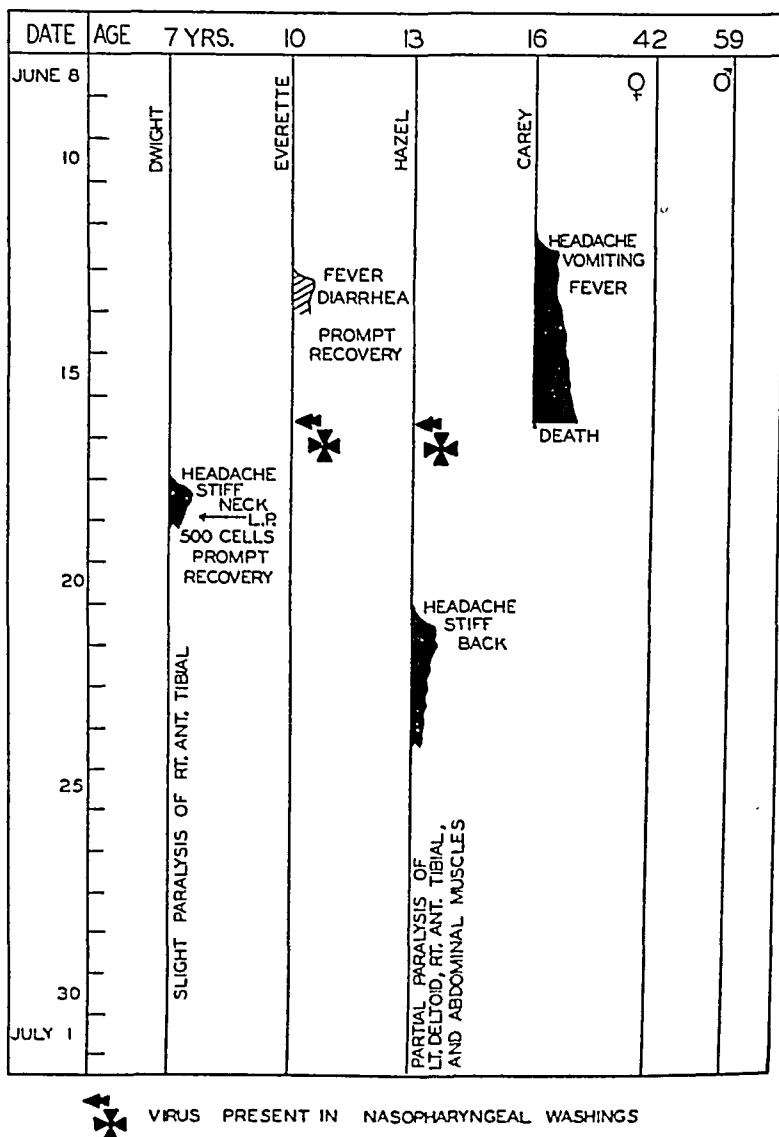
On the other hand there is a single noteworthy example of the successful isolation of the virus by Taylor and Amoss (14) from the throats of two members of a poliomyelitis family including (a) a case of minor illness or abortive poliomyelitis, and (b) a child, 5 days prior to the development of a mild case of frank poliomyelitis. In their family study the clinical events were so similar to many of those in our recent experiences that they will be transcribed from their paper in some detail.

The family which lived in the village of Waitsfield, Vermont, consisted of the parents and four children (see Text-fig. 1). On June 12, 1917, the oldest boy, Carey, developed poliomyelitis with initial symptoms at first thought to be those of a gastrointestinal upset. Extensive paralysis was present on the 4th day of the disease and death occurred on this date. On June 13 the next boy, Everette, developed what was probably a characteristic minor illness, marked by fever and diarrhea. He recovered quickly and, subsequently on minute examination, showed no muscular weakness. The youngest child, Dwight, became ill on June 18, and the girl, Hazel, on June 21. Both of these illnesses proved to be mild attacks of poliomyelitis, although, if it had not been for the fact that particular attention had been attracted to this family by the presence of a fatal case of poliomyelitis in the oldest member, it seems questionable whether the other cases would have been readily diagnosed. In other words it is conceivable that they could have passed as examples of "summer gripe," etc., had not careful examinations been done in both instances.

Nasopharyngeal irrigations were obtained from two of the children,—Everette and Hazel,—on June 16; *i.e.*, 4 days after the former had developed symptoms of a minor illness, and 5 days before the latter developed symptoms of a mild attack of poliomyelitis. The material obtained was treated in the following manner. Distilled water was used as the irrigating medium, 60 cc. for Everette, and 100 cc. for Hazel. To the sample washings 10 per cent of ether was added; they were then shaken with glass beads for 2½ hours, and centrifuged at high speed for 2½ minutes. The supernatant fluid was passed through a Berkefeld N candle and concentrated to 2 cc. *in vacuo* at 35°C. This amount was injected intracerebrally into two *rhesus* monkeys (A—Everette, and B—Hazel) within 6 hours after the collection of the specimens.



Both monkeys developed paralyses. Monkey A recovered, its sera was subsequently shown to be capable of neutralizing poliomyelitis virus, and the animal itself was resistant to the reinoculation of a large dose of virus. Monkey B was sacrificed on the 9th day after inoculation. The histological picture of the spinal cord and medulla was that of poliomyelitis, and emulsions of these organs were capable of inducing the disease in another monkey.



TEXT-FIG. 1. Schematic diagram of a family study reported by Taylor and Amoss (14). The vertical lines represent individual members of the family; their respective ages appear at the top. The solid areas roughly indicate the clinical course of an attack of poliomyelitis; the shaded area, the course of a minor illness.

The observations quoted above are of great importance. Here again the number of times that attempts of this kind have been repeated, and the number of unsuccessful attempts to isolate the virus from cases with mild or abortive poliomyelitis either by these or other workers is unknown, but the reported successes are sufficiently small to suggest that its isolation from such cases is not easy.

### *Methods*

Prompted by the results of Taylor and Amoss (14) we followed somewhat the same procedure in our own attempts to isolate the virus from examples of characteristic minor illnesses occurring coincidentally either within a family, or within a small community in which poliomyelitis was present. In general the following procedure was employed.

*Preparation of Material for Inoculation.*—The patient was instructed to gargle the throat with 25 to 125 cc. of sterile distilled water, or it was washed through the nasopharynx by means of a syringe inserted into the nares. The washings thus obtained were transferred to a flask containing glass beads, 10 per cent of ether was added, and the flask was shaken for 15 minutes. The material was then usually concentrated by vacuum distillation at 37°C. for a period of from 4 to 7 hours,—by this procedure we were seldom able to reduce the washings to less than a fourth of their original volume. To the concentrated washings phenol was added to make a concentration of 0.5 per cent and the material was allowed to stand for  $\frac{1}{2}$  hour. This step in the procedure was employed in preference to filtration because of the experiences previously reported by Flexner and Clark (9); it has also been subsequently shown that the virus may remain active in this concentration of phenol for many months (15).

All inoculations were done under ether anesthesia. One monkey was employed for each of the tests, which were done by the intracerebral route, using between 0.8 to 1.2 cc. of the concentrated, phenolized washings as the amount of inoculum. Bidaily temperature readings covering a period of 3 weeks were taken on all monkeys thus inoculated. Of the twenty monkeys employed for the experiment, sixteen were *Macacus rhesus*, four were of other varieties listed in Table I. One monkey inoculated with material from a healthy contact succumbed on the 6th day from a brain abscess and has not been included in the table.

Various alterations were made from time to time in our methods of obtaining and treating the washings prior to their inoculation. These consisted in (a) attempts to reduce the volume of the irrigating fluid by washing about 30 to 40 cc. of saline solution through and through the nasopharynx; (b) the concentration of the material by ultrafiltration through collodion sacs. In the latter procedure we were guided by the belief that the virus was not diffusible through the collodion mem-

branes we employed. It may suffice to say, however, that the procedure first outlined was the one in which our two positive results were obtained.

### *Selection of Cases*

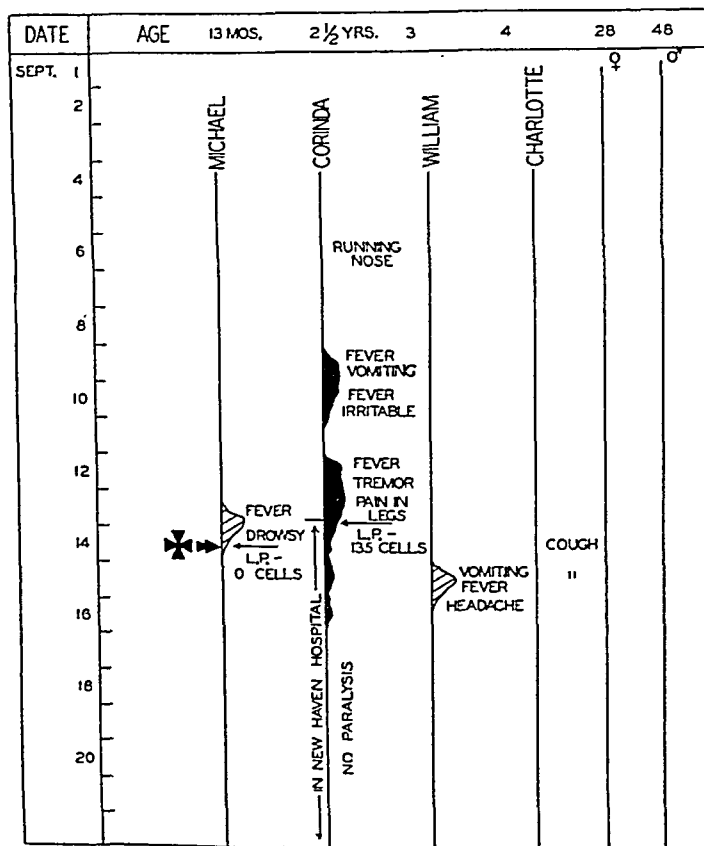
It was our object to select and test oral washings on patients who developed a characteristic minor illness (comparable to one of Wickman's abortive types), under circumstances in which known exposure to a case of poliomyelitis had existed; and to compare the results with findings similarly obtained from known patients with poliomyelitis, and from healthy contacts. Text-figs. 2, 3, and 4 are sample diagrammatic representations of some of the situations under which our washings were obtained.

In Text-fig. 2 is shown a family with four children,—Family Rn. In this family a frank case of poliomyelitis occurred which was followed closely by two cases of characteristic minor illness in two other children. In one of the latter, the most prominent symptoms were fever and drowsiness; in the other, vomiting, fever, and headache. Both of these children were well within 36 hours of the onset of symptoms. In the youngest child (Michael), however, the symptoms were sufficiently suspicious to warrant a diagnostic lumbar puncture which proved negative. At the time the puncture was done nasopharyngeal washings were also obtained.

In Text-fig. 3 is shown another family,—Or. In this family which is composed of six children, there occurred one case of frank poliomyelitis (Irving); one suspected case (Frances); and two examples of characteristic minor illness (Evelyn and Charles). The series of small black arrowheads, which appear as legends in this figure, indicate that on September 16, washings were obtained from the child Irving, supposedly on the 1st day of an attack of poliomyelitis; two children Evelyn and Charles, who were on the 3rd day of a minor illness; and one child Edward, who was presumably a healthy contact.

In Text-fig. 4 a diagram is shown portraying events in one of the communities in which similar studies were made. This community consisted of a summer colony situated on the shore of Long Island Sound in fairly close proximity to New Haven. All of the juvenile members of the group have been portrayed as vertical lines, denoting by their length the period of time in which they were members of the

roup. Illnesses have been recorded by the legends used in the previous figures. It will be seen that the population was not fixed, in that families were constantly moving in or out. During late July and early August several cases of mild illness, characterized by sore

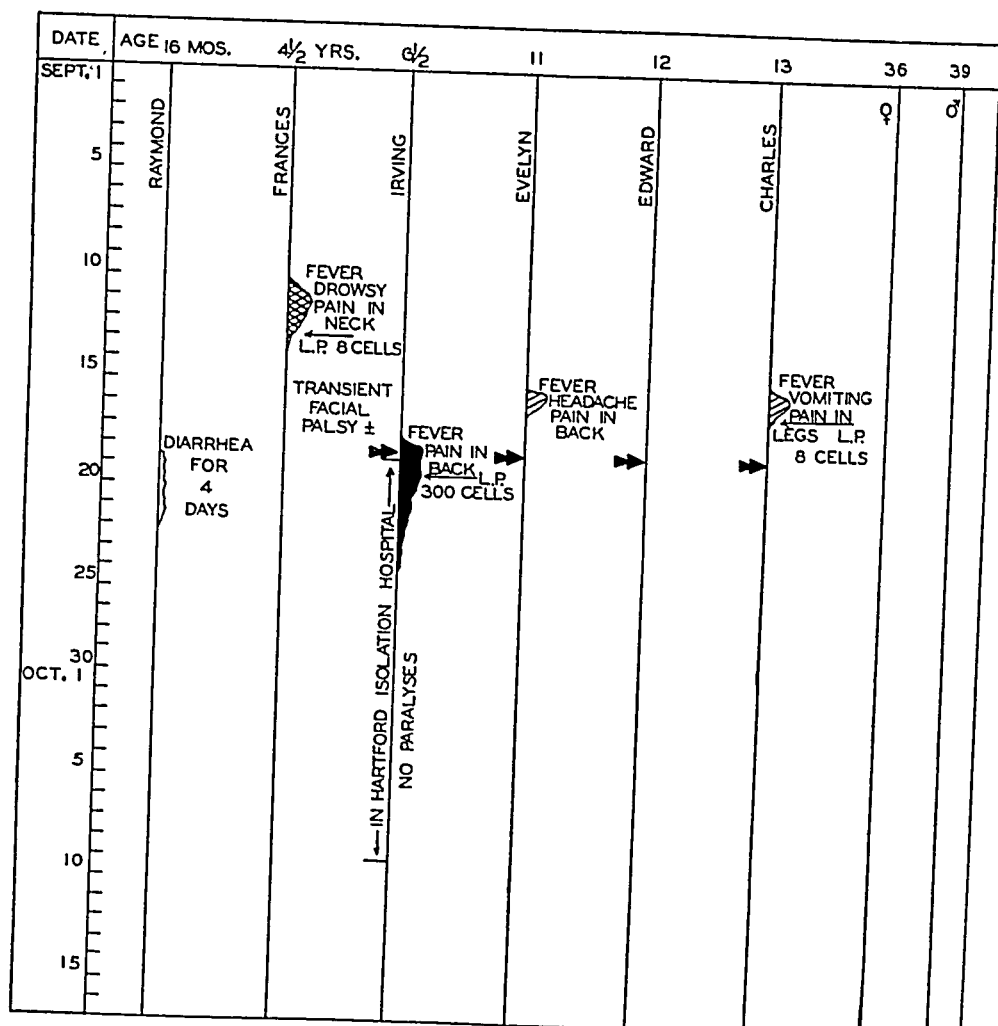


✠ ➡ VIRUS PRESENT IN ORAL WASHINGS

TEXT-FIG. 2. Diagram of Family Rn. The legends are the same as those in Text-fig. 1.

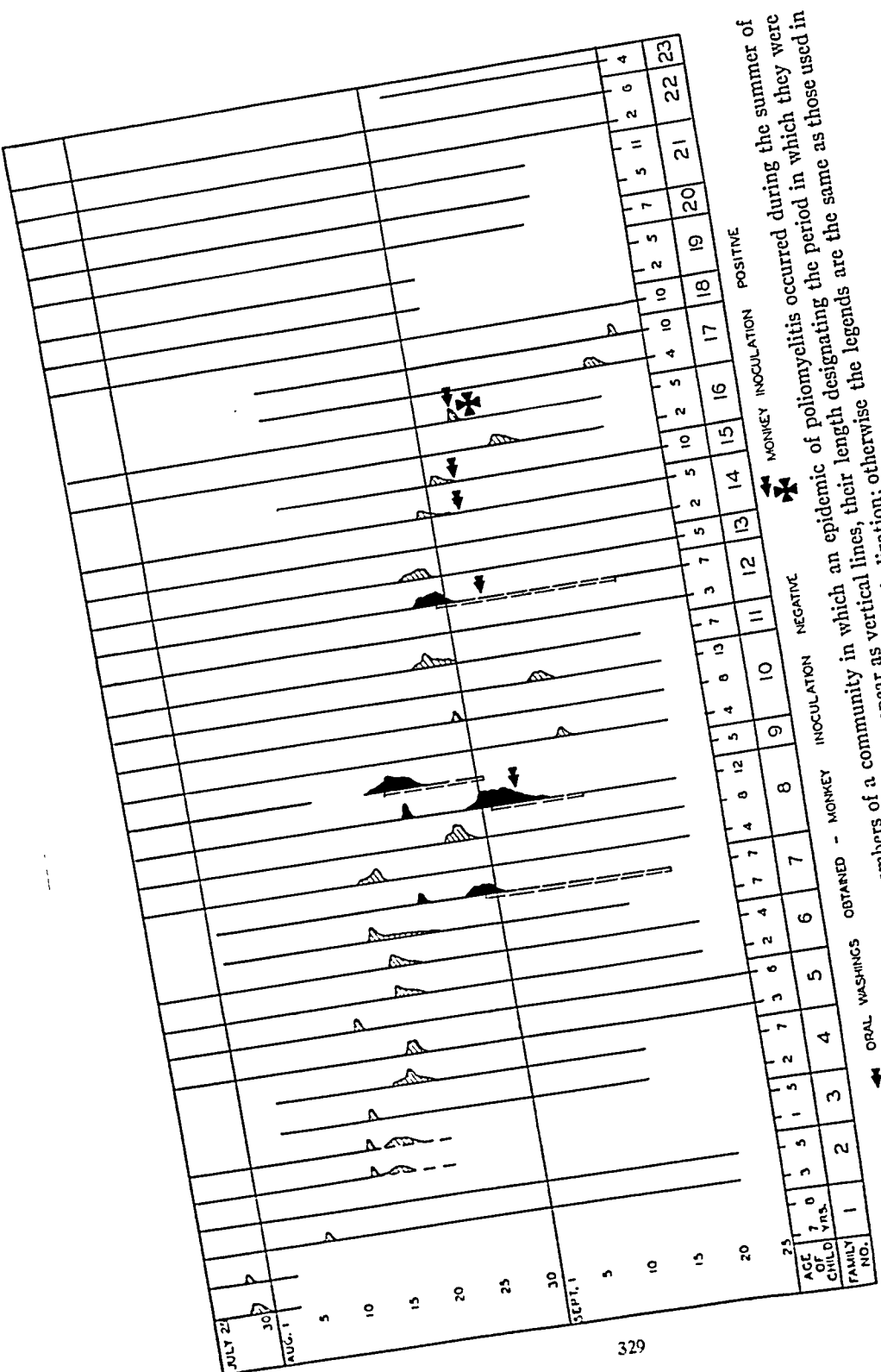
throat, fever, vomiting, and headache appeared among the children, but although there was considerable apprehension by attending physicians and the community itself, as to whether these illnesses might be poliomyelitis, symptoms did not appear at first from which

a definite diagnosis could be made, and for want of a better term they were labelled "summer gripe." However, on August 20 a child from Family 9, who had left the summer colony the week before, developed poliomyelitis, and shortly thereafter three other cases



TEXT-FIG. 3. Diagram of Family Or. The black arrowheads indicate the time at which oral washings were obtained.

appeared among members of the colony in rather rapid succession. Meanwhile the "summer gripe" continued. Three out of seven children who contracted it during September, had arrived in the community subsequent to August 15. In all, therefore, out of 41 children



TEXT-FIG. 4. Diagram of the juvenile members of a community in which an epidemic of poliomyelitis occurred during the summer of 1931. All of the community children under 15 years of age appear as vertical lines, their length designating the period in which they were members of the community. The double set of divided lines indicates hospitalization; otherwise the legends are the same as those used in the previous diagrams.

in the colony there were 29 instances of acute illness between July 30 and September 22. Four of these illnesses were examples of frank poliomyelitis. Many of the others merely consisted of fever, headache, and vomiting lasting for only a day; but some were of several days' duration; others presented two distinct phases, and not a few offered real problems in the differential diagnosis of frank poliomyelitis. It will be seen in Text-fig. 4 that five oral washings were obtained from this group. They include two cases of poliomyelitis (one of which was in the convalescent stage), representing Families 8 and 12 respectively; three children with so called "summer grippe" at various stages of the disease, representing Families 14, 15, and 16; and one healthy contact, a 16 year old nurse girl, who, owing to her age, does not appear on the chart. The virus was detected in one of these washings, namely, the older child from Family 16 whose throat was irrigated within a few hours after he had come down with an attack of fever and vomiting on September 4. He was symptom-free within 24 hours of the onset of his illness.

#### RESULTS

From a series of twelve attempts to isolate the virus in early and late stages of these characteristic minor illnesses in association with poliomyelitis, we were successful in two instances.

The circumstances under which the washings were obtained have already been given in Text-figs. 4 and 2 respectively. A brief account of the actual clinical events which transpired in these cases was as follows:

Robert We., aged 5, (*cf.* Text-fig. 4) had been exposed during the period of Aug. 20 to Aug. 29 to three cases of poliomyelitis. At noon on Sept. 4 he became ill with fever and vomiting; these were the symptoms which many children in this community had had during the preceding 6 weeks. At 5:00 p.m. on Sept. 4, oral washings were obtained. This material was inoculated into Monkey 22 that same evening. The child was well by the following morning. The monkey developed the experimental disease.

Michael Rn., aged 13 months, (*cf.* Text-fig. 2) had been exposed to his sister Corinda, who was admitted to the New Haven Hospital suffering from poliomyelitis on Sept. 13. She was then in the 6th day of her disease. On the same day Michael became ill with fever and drowsiness and on the evening of the following day he was brought to the New Haven Hospital under the suspicion that he also

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might have poliomyelitis. A lumbar puncture was done at this time, which showed no cells and a negative test for globulin by the Pandy test. Oral washings were obtained at this time. The child was well by the following morning,—Sept. 15. The washings were inoculated into Monkey 29, which subsequently developed the experimental disease and will be described later.

Our results have been analyzed in a number of ways. The first analysis appears in Table I. Here are included data which bear on the evidences of exposure to poliomyelitis in the cases studied; the results of lumbar punctures made on the day on which most of the washings were obtained; methods and technique of obtaining and treating the washings; and the immediate or subsequent fate of the monkeys employed. The experiments were run in three sets, in each of which we attempted to include (a) cases of characteristic minor illness with exposure to poliomyelitis, (b) frank cases of poliomyelitis, (c) contacts. Successful results in obtaining the virus were encountered only in the first set, and only with the characteristic minor illnesses. In none of the three cases of frank poliomyelitis did we succeed in isolating the virus, and in none of the contacts.

A further analysis has been made which concerns the day of the disease on which the washings were obtained. This appears in Table II. Here it will be readily seen that our two successful attempts to isolate the virus from the minor illness cases occurred from material obtained on the 1st and 2nd days of the disease; that a single attempt made on the 1st day was successful, and that one out of four attempts made on the 2nd day was successful; the remaining seven attempts made on subsequent days were all negative. There is, however, a further point, best shown in Text-fig. 5 which may be of some significance; namely, that of the four irrigations done on the 2nd day of the disease, two were obtained while the patient was still having symptoms, and two after symptoms had subsided. One of the two having symptoms on the 2nd day of his minor illness harbored the virus, while from neither of the two whose symptoms had subsided was the virus demonstrated. It is unfortunate that we were unable to isolate the virus from oral washings obtained from the three frank cases of poliomyelitis. In two of them the irrigation was probably done too late in the disease, but in one (see Text-fig. 3) the circumstances should have been satisfactory for its detection.



No. of experiment	Patient	Clinical aspects of case				Method of collecting material for inoculation
		Age	Evidence of exposure to poliomyelitis	Day of illness	No. of cells in spinal fluid	
Experiment 1, started Sept. 4, 1931	Minor illnesses	yrs.				
	We.	5	4 nearby cases	1	Not tested	Throat rinsed with water
	Rn.	1	Case in family	2	0	Throat and nose irrigated with water
	Bt.	7	4 nearby cases	2	Not tested	Throat rinsed with water
	Pn.	10	4 " "	4	" "	" "
	Ld.	5	4 " "	5	" "	" "
	Ln.	5½	4 " "	13	0	" "
	Poliomyelitis cases					
	Ks.	7		9	38	Throat rinsed with water
	Gn.	12		13	91	" "
	Contact L. N.	16	Taking care of case		Not tested	" "
Experiment 2, started Sept. 20, 1931	Minor illnesses					
	Ev. O.	11	2 cases in family	4	Not tested	Throat rinsed with saline
	C. O.	13	" "	4	8	" "
	Poliomyelitis I. O.	6½		1	300	Throat rinsed with saline
Experiment 3, started Oct. 3, 1931	Contact E. O.	12	2 cases in family		Not tested	" "
	Minor illnesses					
	R. B.	6	In New Haven, fall, 1931	3	4	Throat and nose rinsed repeatedly with saline
	P. L.	7	" "	2	4	" "
	E. M.	6	" "	2	3	" "
	E. W.	11	In Westport, Conn., fall, 1931	3	5	" "
	Contact Sister of P. L.	10	In New Haven, fall, 1931		Not tested	" "
	Brother of E. W.	7	In Westport, Conn., fall, 1931		" "	" "

\* This monkey together with several others of the same species was probably suffering from



*Evidences of Poliomyelitis in the Monkey.*—It is pertinent to recall at this point that the intracerebral inoculation of human or monkey material containing the virus of poliomyelitis does not invariably produce the disease in the monkey (9, 16), and that the experimental disease induced in monkeys by human strains is considerably milder than that obtained with so called fixed monkey virus. We were, therefore, fortunate in obtaining what appear to be definite results in our two positive monkeys.

TABLE II  
*Summary of Results of Monkey Inoculations with Oral Washings*

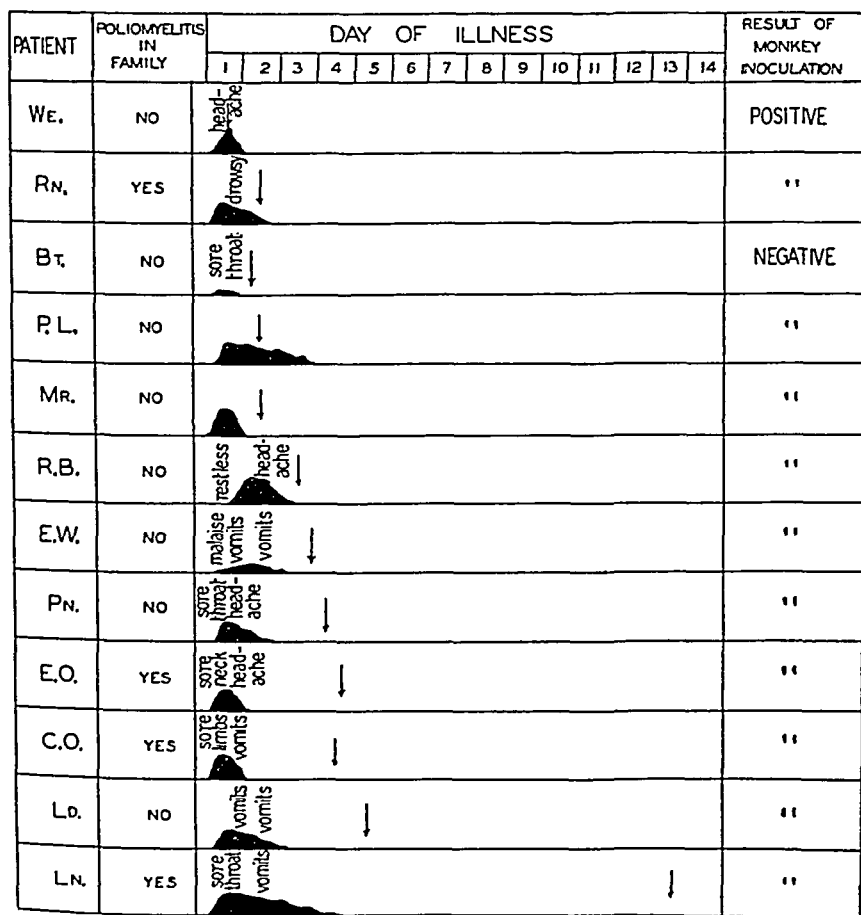
	Day of illness or contact on which washing was done							Total
	1	2	3	4	5	6-10	11-15	
Minor illness								
No. of cases.....	1	4	2	3	1	1		12
No. of positive results.....	1	1	0	0	0	0		2
Contacts								
No. of subjects.....		1*	1*	1*				3
No. of positive results.....		0	0	0				0
Poliomyelitis								
No. of cases.....	1					1	1	3
No. of positive results.....	0					0	0	0
Contacts								
No. of subjects.....	1*							1
No. of positive results.....	0							0

\* The day of contact represents the day numbered from the onset of the last case to which the subject was intimately exposed.

Four criteria have been used as evidence of poliomyelitis in the inoculated monkeys: (a) the temperature curve; (b) the development of paralysis; (c) the findings at autopsy; and (d) the passage of the disease to other monkeys.

*Temperature Curves.*—The characteristic temperature curve which is exhibited by monkeys infected with standard strains of poliomyelitis virus has been described by Kramer, Hendrie, and Aycock (17). These authors call attention to the fact that there may be an immediate

brief rise in temperature following inoculation, but this usually does not last beyond the first 24 hours. The incubation period or period



↓ = ORAL WASHINGS TAKEN

TEXT-FIG. 5. Diagram showing twelve examples of minor illnesses from which oral washings were obtained. The solid areas roughly indicate the course of the disease as reconstructed from temperature readings.

of normal temperature prior to the onset of the true experimental disease generally lasts 4 to 8 days, but at times as long as 18 to 21 days. With the onset of the disease there is an abrupt rise in tem-

perature reaching 104–107°F. Fever may persist from 1 to 3 days before the onset of the usual recognizable symptoms, such as paralysis. With the appearance of these symptoms the temperature is already declining and when paralysis is extensive there may be a pronounced drop to subnormal values.

In Text-fig. 6 are shown the temperature charts of the two monkeys in which the experimental disease was produced. The experimental protocols read as follows:

Monkey 22—Patient We. 20 cc. of oral washings were obtained on Sept. 4, 0.5 per cent phenol was added, and 1.2 cc. was inoculated intracerebrally a few hours later on the same day. For 12 days the temperature fluctuated between 101° and 102.6°. On the 13th day it rose to 104° and fluctuated between 102° and 104° for 4 days, falling abruptly to subnormal on the 17th day. It is possible that paralysis may have developed at this time but it was not definitely noted until 3 days later when it became evident the animal was not using the right arm, and that the right leg was weak. The animal was sacrificed on the 27th day. The autopsy revealed typical lesions of poliomyelitis in the spinal cord (*cf.* Fig. 1).

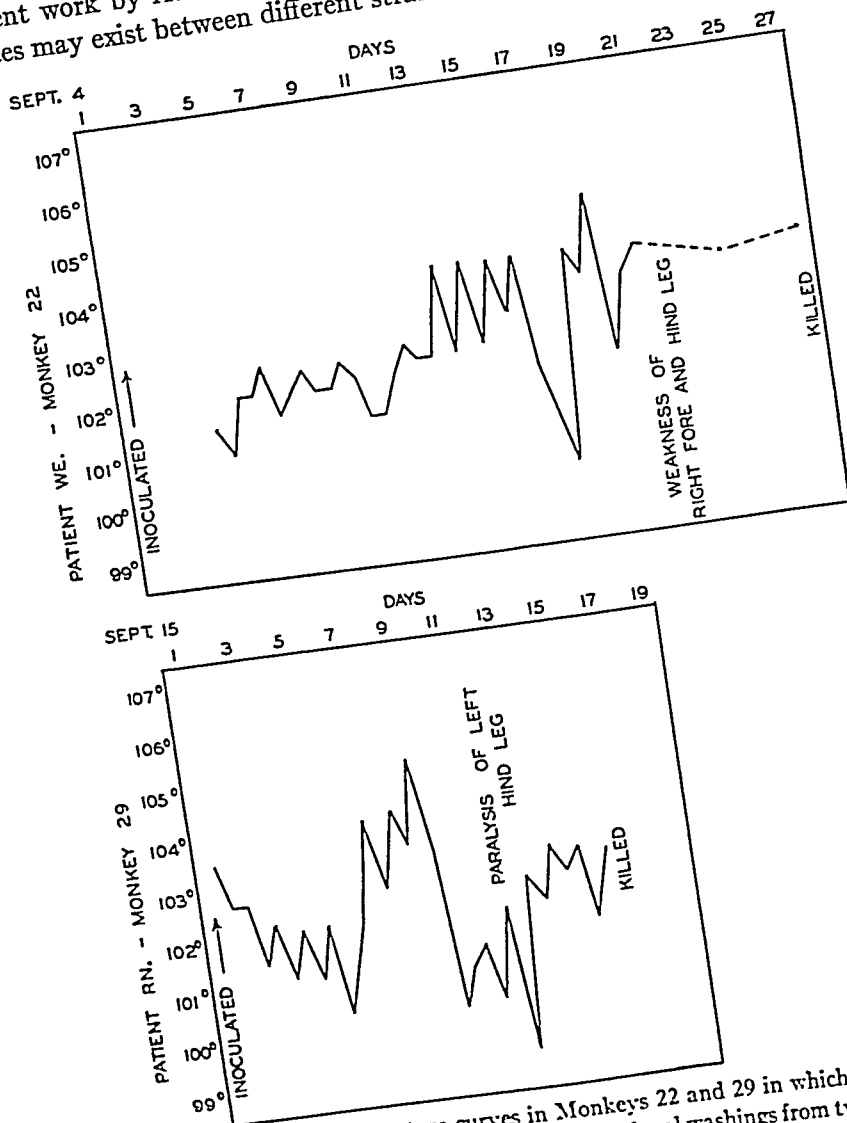
Monkey 29—Patient Rn., 100 cc. of oral washings were obtained at 10:00 p.m. on Sept. 14 to which 10 per cent of ether was added and the material was left in the ice box overnight. On the following day it was concentrated to one-fifth its original volume by vacuum distillation over a period of 5 hours at 37°C.; 0.5 per cent phenol was added; and 1 cc. of the material was inoculated intracerebrally into this monkey at 3:00 p.m. Sept. 15. The monkey's temperature gradually fell over a period of 6 days and then underwent a rapid rise of 3–4°. This febrile period lasted 4 days, to be followed by a sharp drop on the 10th day after inoculation, with an irregular return to normal values. On the 12th day it was first noted that the animal dragged the left hind leg. Subsequent examinations revealed complete paralysis of this limb. The animal was sacrificed on the 17th day. The autopsy revealed typical lesions of poliomyelitis in the spinal cord (*cf.* Fig. 2).

Several of our other monkeys, which failed to show evidences of the experimental disease, were sacrificed and studies of the tissues were made. In none of these did we find evidences of poliomyelitis (*cf.* Table I). Practically all of the remaining, surviving monkeys were subsequently tested and found susceptible to infection with a standard strain of poliomyelitis virus.<sup>3</sup> It seems pertinent to add,

<sup>3</sup> We are indebted to Drs. W. H. Park and E. R. Weyer of the Bureau of Laboratories, Department of Health, City of New York, for this strain of virus. It is a mixed strain derived from several sources.

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however, that the latter procedure may not be as satisfactory a test for immunity to poliomyelitis in the monkey as was once believed, for recent work by Australian investigators has shown that wide differences may exist between different strains of the virus (18).



TEXT-FIG. 6. Temperature curves in Monkeys 22 and 29 in which the disease was produced by the inoculation of oral washings from two of characteristic minor illness.

*Autopsy Findings.*—As stated in the preceding protocols, tissues from the spinal cord and brain of Monkeys 22 and 29 revealed lesions characteristic of poliomyelitis. Both animals were sacrificed 5 days after paralysis was first noted. The lesions are shown in Figs. 1 and 2.

*Passage Experiments.*—Both strains We. and Rn., isolated from oral washings as above described, were subjected to subsequent monkey passage. Here we encountered the usual problems which arise in attempting to establish in the monkey, recently isolated human strains, for the experimental disease so produced is often not easy to detect and may not fulfil all of the usually recognized diagnostic criteria: (a) characteristic temperature reactions; (b) paralysis; and (c) the presence of typical histological lesions in the spinal cord or medulla, that is to say lesions which include the characteristic, perivascular, round cell infiltrations. All of our positive diagnoses have been based upon the last criterion; *i.e.*, histological lesions in the central nervous system.

To facilitate the early passage of these strains recourse was had to the method of double inoculation (9). The results of the early passage experiments appear in Text-fig. 7.

With the We. strain the first-passage monkey (No. 67) received two inoculations of a 10 per cent suspension of spinal cord intracerebrally and intraperitoneally. It is probable that the second inoculation was superfluous to establish the infection. There were certain unusual features about this monkey in that the animal had diarrhea, and on the 6th day after inoculation the limbs and face became edematous. Subsequently it became quite ill, weak, and refused to move about. The edema practically disappeared by the 8th day at which time a second inoculation was done. The animal died the next day. Unfortunately, temperature readings were discontinued during the edema period but on the day of death the temperature was found to have fallen to a subnormal value. Extensive lesions typical for poliomyelitis were found in sections of the cord in spite of the fact that the development of actual paralysis during the last few days had not been noted although the animal had been too weak for an adequate examination. The second-passage monkey (No. 35) received a single intracerebral and intraperitoneal inoculation of a 5 per cent suspension and developed the experimental disease with unusual rapidity. Subsequently this strain has been carried on through further monkey passages.

Two first-passage monkeys were inoculated with the Rn. strain. One monkey (No. 66) received only one inoculation, and, starting on the 6th day developed

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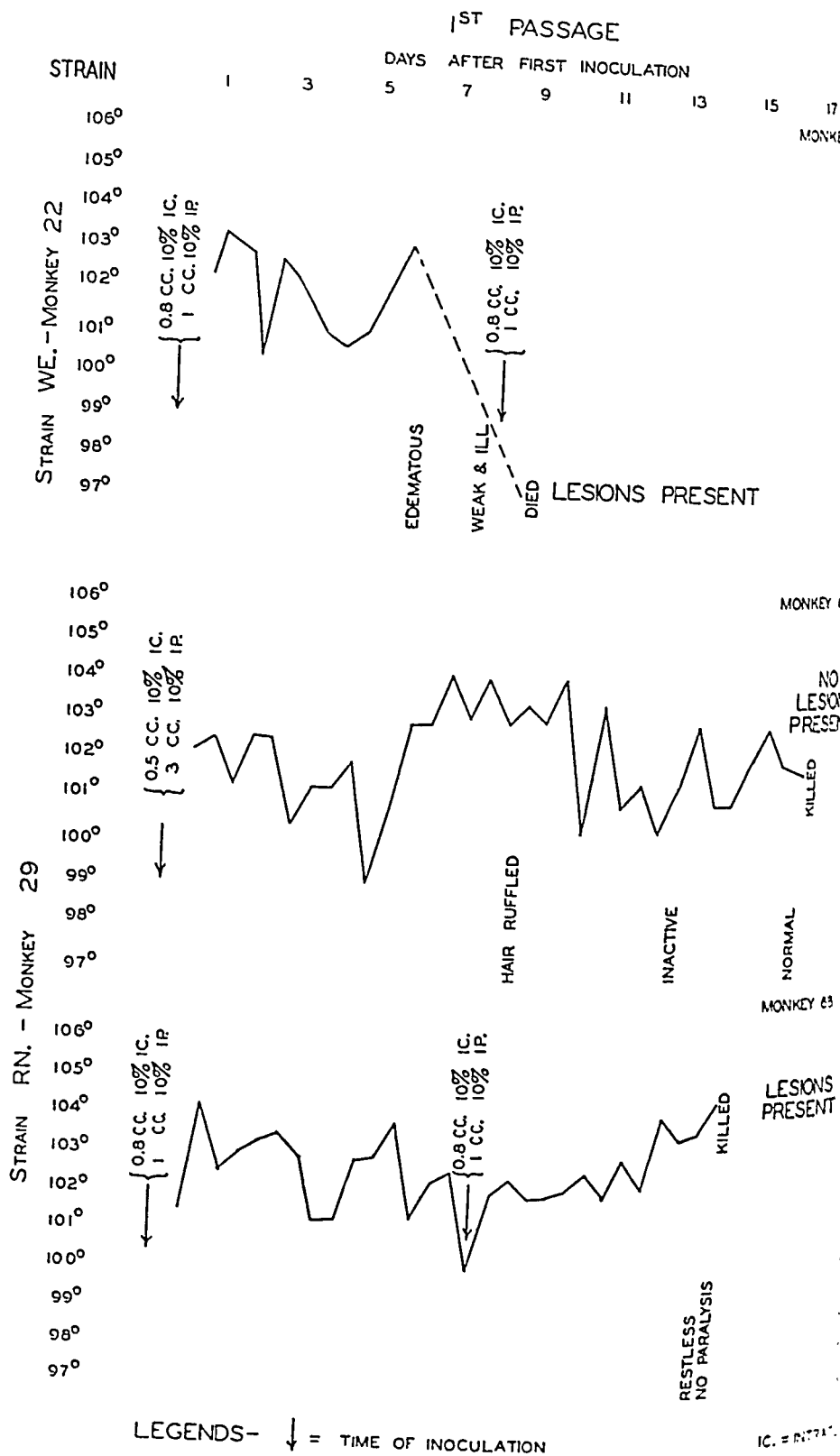
fever which ran a course comparable to that which we have seen in experimental poliomyelitis. With the fall in temperature no paralysis was detected and subsequently at autopsy the examination of the central nervous system proved negative. The other first-passage monkey showed no elevation of temperature by the 7th day and was then given a second inoculation. The temperature rose 6 days later and the animal was killed. Paralysis had not been observed, but it is possible that it would have developed had the animal been spared a few more days. Typical lesions of poliomyelitis were present in the cord and medulla. In the second-passage monkey which was inoculated with material from Monkey 68, these events were practically reduplicated, only in this animal the experimental disease was allowed to run for a longer period. Fever developed 5 days after the second inoculation, persisted irregularly for 6 days, and subsequently fell with the development of paralysis. Typical lesions of poliomyelitis were found at autopsy.

The results show that both strains We. and Rn. could be subjected to monkey passage. We became impressed early in this phase of the work, however, with the differences which exist between the experimental disease produced by strains recently derived from human sources and the experimental disease produced by an established or fixed monkey strain. In the latter so called missed and abortive cases of the experimental disease have been described (16), and we are inclined to believe that these may be more frequent in recently isolated strains and that here examples of actual infection with the poliomyelitis virus without the development of all the usual unequivocal signs, particularly without the development of typical paralysis, may be common. The number of experimental observations which we have at present are insufficient as yet for a statistical analysis of this point.

#### COMMENT

In corroboration of the beliefs held by many previous observers, and in confirmation of experimental proof obtained 15 years ago (14), evidence has been brought in this paper to suggest that a common causal relationship exists between certain characteristic minor illnesses and the usually accepted entity of clinical poliomyelitis. This evidence consists in the detection of the virus of poliomyelitis in the throats of two patients during the early or mid stage of a characteristic minor illness, who, although seen by several physicians, were not thought to be suffering from either frank poliomyelitis or the





TEXT-FIG. 7. Temperature curves exhibited by the first- and second-passage monkeys (We.) and 29 (RN.) and 63 (RN.).



abortive form of the disease in its usually accepted sense. We are aware of the possibility that the presence of the virus in the throats of two children who had been exposed to poliomyelitis, may indicate that they were carriers of the virus and not necessarily suffering from infection with it. The consistency with which we encountered negative findings among contacts and also those who had had these characteristic minor illnesses but were convalescent, would, however, militate against this view.

The question as to whether such characteristic minor illnesses are actually examples of poliomyelitis or not would seem to be one of definition. The answer involves an appreciation of the complex adjustments occurring between host and incitant, which may be expressed by different reactions in the former; such as, severe, mild, or absent clinical manifestations of disease. The elucidation of this problem must rest upon further observation and experiments, particularly upon the nature of immunity in this disease. Nevertheless the evidence at hand is sufficient to suggest that, since these characteristic minor illnesses outnumber by many times examples of frank poliomyelitis, their rôle as far as the spread of the disease is concerned is, indeed, important.

#### CONCLUSIONS

Experiments are reported which describe the isolation of poliomyelitis virus from the throats of two patients during an attack of so called abortive poliomyelitis (Wickman type), or what we have termed characteristic minor illnesses in association with poliomyelitis.

This finding represents added evidence in favor of the belief, previously held by many observers, that certain types of minor illness, which accompany an epidemic of poliomyelitis, probably represent mild cases of the disease.

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## EXPLANATION OF PLATE 19

- FIG. 1. A section of spinal cord from Monkey 22.  $\times 75$ .  
 FIG. 2. A section of the spinal cord from Monkey 29.  $\times 75$ .







# HISTOLOGICAL STUDIES ON HOG CHOLERA\*

## II. LESIONS OF THE VASCULAR SYSTEM

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PLATES 20 TO 22

(Received for publication, April 22, 1932)

As a part of our study of the histological changes associated with the hog cholera virus a description of the findings in the central nervous system has been published (1). In this description attention was called to the lesions in the blood vessels. As the study progressed the importance of these blood vessel lesions became more evident and the present paper is a summary of our findings in this connection.

### LITERATURE

Proescher and coworkers (2, 3) found in some organs an acute endangitis combined with a thromboangitis and an end arteritis. Bel (4) described hyperemia of capillaries from which he considers the hemorrhages to be derived but does not refer to special capillary lesions. Roehrer (5) whose work has been done simultaneously with but independently of our own, concludes from the study of the lymph nodes and spleen that the hog cholera virus primarily affects the walls of capillaries and smaller arteries and that other lesions in these organs, such as hemorrhages and necrotic foci, are due to these blood vessel changes. Dide (6) failed to find any characteristic lesions in hog cholera except in the brain. These lesions were similar to those described by Seifried (1) and by Roehrer (5). Eberbeck (7) made no detailed study of the blood vessel system.

### Material and Methods

The material for this study is part of that used for the work on the lesions in the central nervous system. Thirty cases of hog cholera were used of which twenty-two were infected by intramuscular injection or by contact and eight were

\* Presented in part at the meeting of the American Veterinary Medical Association in Kansas City, Missouri, August 25 to 28, 1931; *J. Am. Vet. Med. Assn.*, 1932. 33, 225.



field cases. The experimentally infected animals were killed with chloroform or died 6 to 32 days after inoculation or contact infection. All infected animals had characteristic temperatures and the majority showed more or less typical lesions at autopsy. A few animals, including most of the field cases, did not exhibit lesions that were definite enough to establish a diagnosis. The tissues of a number of cases were cultured immediately after autopsy in order to determine whether secondarily invading bacteria were present.

*Capillaries and Precapillaries.*—In practically all of the organs we studied these vessels usually show the earliest and most pronounced lesions. However, within an individual organ not all capillaries are equally involved. In some organs, for example the kidneys and skin, there is undoubtedly a new formation of capillaries which may be distended with red blood corpuscles or may be empty and collapsed. This is especially true in the lymph nodes. In many cases an enormous swelling and faint staining of the capillary wall is conspicuous even under low power (Fig. 1). With higher magnifications the nuclei of endothelial cells appear markedly enlarged, and because of their lack of chromatin they remain pale or practically unstained (Figs. 2, 4). Frequently the endothelial cells are thrown off or are even entirely lost. Various stains bring out the pale, homogeneous, and hyaline-like appearance of the remaining parts of the blood vessel wall. The thin layers of collagenous or reticular fibers, which closely surround the endothelium, are only partly visible and are undergoing the same changes. The condition is evidently necrotic. It may be so advanced as to change the blood vessel into a thickened hyaline tube, in which cellular or fibrillar elements are no longer visible, or it may lead to a partial or complete occlusion of the capillary lumen (Fig. 3). Less frequently there is a circumscribed proliferation and tumor-like multiplication of endothelial cells, which also show retrogressive changes. In addition to these lesions, the capillary endothelial cells sometimes show nuclear fragments of various sizes and forms scattered around in the more or less thickened or occluded eosinophilic blood vessel wall (Fig. 3). This necrotic process may be restricted to the capillary wall or may extend into the surrounding tissue. Fat in fine droplets can be demonstrated in both capillary walls and perivascular necrotic areas. Thrombosis rarely occurs in the small blood vessels.

*Small and Medium Sized Arteries.*—Although fundamentally the

lesions of the medium sized arteries do not differ from those of the capillaries, they are more striking in appearance and occur less regularly (Fig. 4). Swelling and proliferation of endothelial cells are common (Fig. 5), and the cells show the same retrogressive changes as do those of the capillaries, either appearing as cell shadows or showing various forms of karyorrhexis (Figs. 6, 7). Both progressive and retrogressive changes may be present simultaneously in an individual vessel. The lesions in the intima are almost regularly accompanied by changes, usually less pronounced, in the other structures of the blood vessel wall (Fig. 7). The findings indicate that the endothelial layer is primarily injured by the virus circulating in the blood stream. The lesions of the media and adventitia consist of a separation and distension of the various elements. This is clearly shown by the elastic tissue stain and various silver methods. The blood vessel wall as a whole is markedly thickened by an edematous condition and a multiplication of cellular elements, particularly in the adventitia. Frequently the artery wall is hyalinized and swollen to such an extent that the lumen is occluded (Fig. 4). In many cases the necrotic condition is indicated by fragmentation of nuclei and a pronounced eosinophilia of the other structures, including elastic tissue fibers and other fibrillar elements. Thrombi in such vessels undergo the same process and frequently the necrosis extends into the perivascular tissue.

Larger arteries are less frequently involved, and the changes in them are mainly restricted to the intima. Occasionally veins also may be affected (Fig. 7), and, even more rarely, lymph vessels.

The vessel lesions just described are found in practically all of the cases studied. Their occurrence, character, and degree of severity vary, however, in different organs. In our material they were most pronounced in the lymph nodes, spleen, and kidneys, while in the central nervous system, liver, intestinal tract, skin, and other organs they were less noticeable. It is sometimes difficult to correlate the gross with the microscopical findings.

#### DISCUSSION

Our study of the vascular system in the various organs reveals the fact that the hog cholera virus, after entering the blood stream, leads to

more or less pronounced lesions of the endothelial cells of the capillaries and other blood vessels and that it also affects the outer layers of the smaller blood vessels and capillaries. It should be noted that the type of these lesions is not directly dependent upon the duration of the disease. Furthermore, there are no fundamental differences between the lesions in pure hog cholera cases and those in cases associated with secondarily invading bacteria. We believe that the magnitude of the vascular lesions depends on the virulence of the virus, and somewhat on the presence of secondarily invading bacteria. In acute cases degeneration of the blood vessel walls is prevalent, while in prolonged cases the proliferative character of the endothelial lesions is more evident. Study of our material has shown that secondarily invading bacteria, such as *B. suispestifer*, *B. pyocyaneus*, and bipolar bacteria, aggravate the necrosis of the blood vessel walls and the resulting lesions. We are convinced that the blood vessel lesions constitute a principal feature in the histopathology of hog cholera and offer a satisfactory explanation for the hemorrhages and foci of necrosis that are characteristic of this disease. The changes resulting from the vascular lesions will be described in a succeeding paper.

The vascular lesions in hog cholera, from a comparative point of view, bear a close resemblance to those in pulmonary blood vessels in human influenza, as described by Oberndorfer (8), Le Count (9), and others.

#### SUMMARY AND CONCLUSIONS

In cases of acute hog cholera the earliest and most pronounced lesions occur in the capillaries and smaller arteries. Larger arteries and veins are less frequently involved. The lesions consist of swelling and proliferation of endothelial cells together with retrogressive changes in them and in the other parts of the blood vessel walls.

The character and degree of these lesions seem to be dependent upon the virulence of the virus and, to some extent, upon the presence of secondarily invading bacteria. We believe that these lesions should be considered a principal feature of the histopathology of hog cholera.

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# EXPLANATION OF PLATES

## PLATE 20

- FIG. 1. Swelling of capillary walls in a lymph node with faintly stained, degenerating endothelial cells. Hematoxylin-eosin.  $\times 315$ .
- FIG. 2. Partial occlusion of small arteries in the kidney by swelling of the endothelial cells and other structures of their walls.  $\times 400$ .
- FIG. 3, *a, b, c*. Cross-section through small blood vessel walls showing various stages of thickening, hyalinization, and nuclear fragmentation (semidiagram).

## PLATE 21

- FIG. 4. Swelling, necrosis, and almost complete occlusion of small arteries in the spleen with coagulation necrosis in the surrounding tissue.  $\times 295$ .
- FIG. 5. Multiplication of endothelial cells in a medium sized artery of a lymph node with retrogressive changes in all other structures of the thickened blood vessel wall. Hematoxylin-eosin.  $\times 300$ .

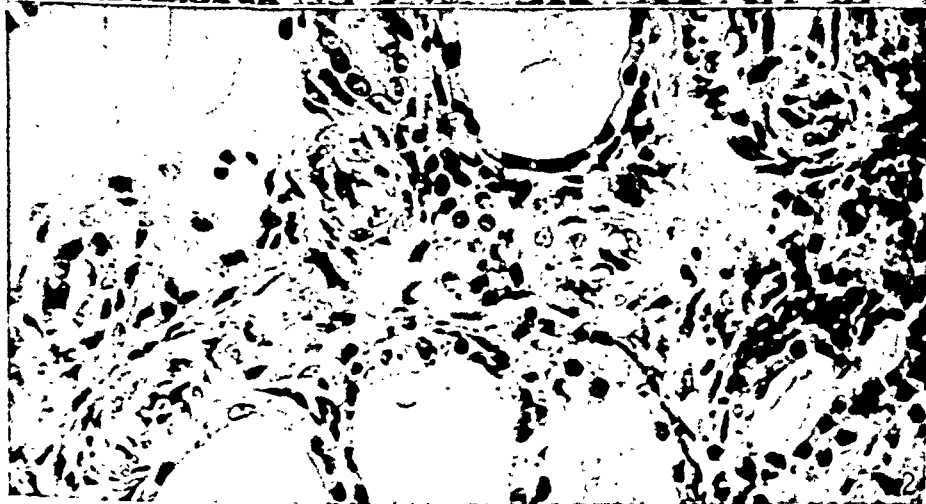
## PLATE 22

- FIG. 6. Destruction of the endothelial cells of a vein and its branch in a lymph node with a marked perivascular necrosis (karyorrhexis figures of various sizes and shapes). Hematoxylin-eosin.  $\times 300$ .
- FIG. 7. Partial destruction of endothelial cells in a vein of the liver. Necrotic process extending into the surrounding tissue.  $\times 300$ .





1



2



a



b



c 3





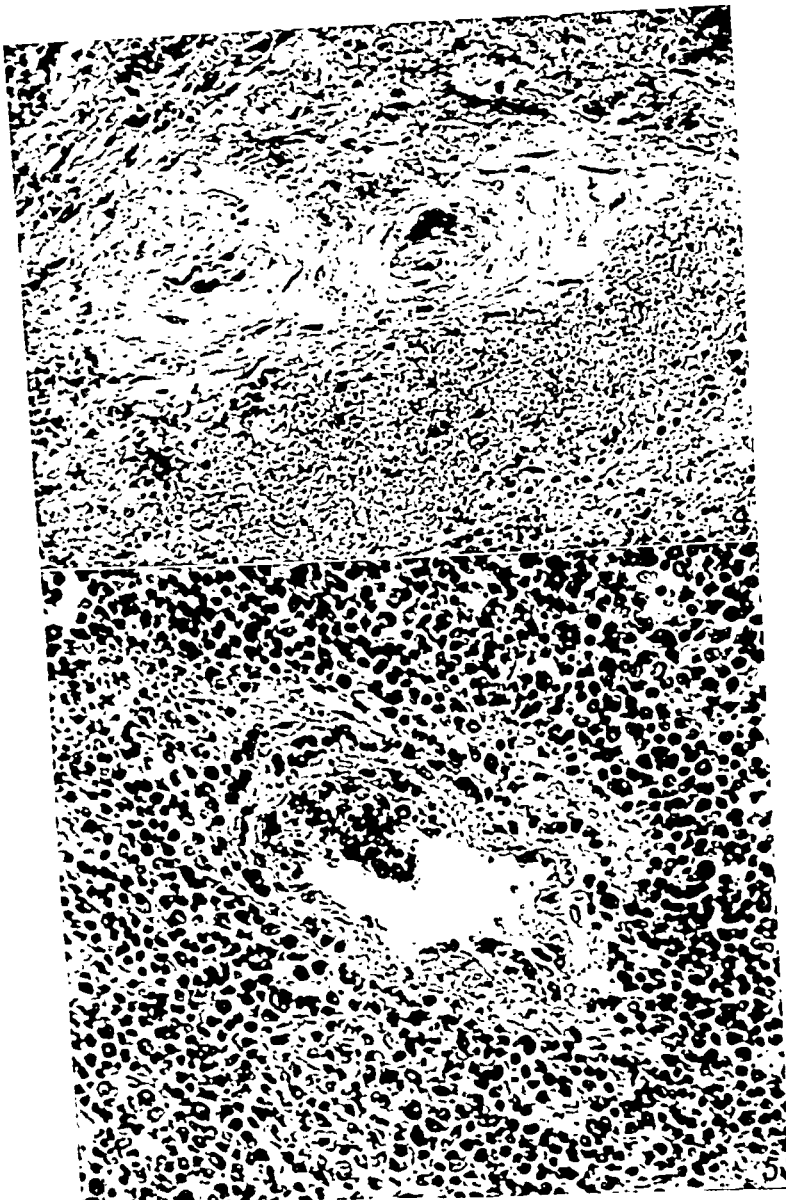
a

b

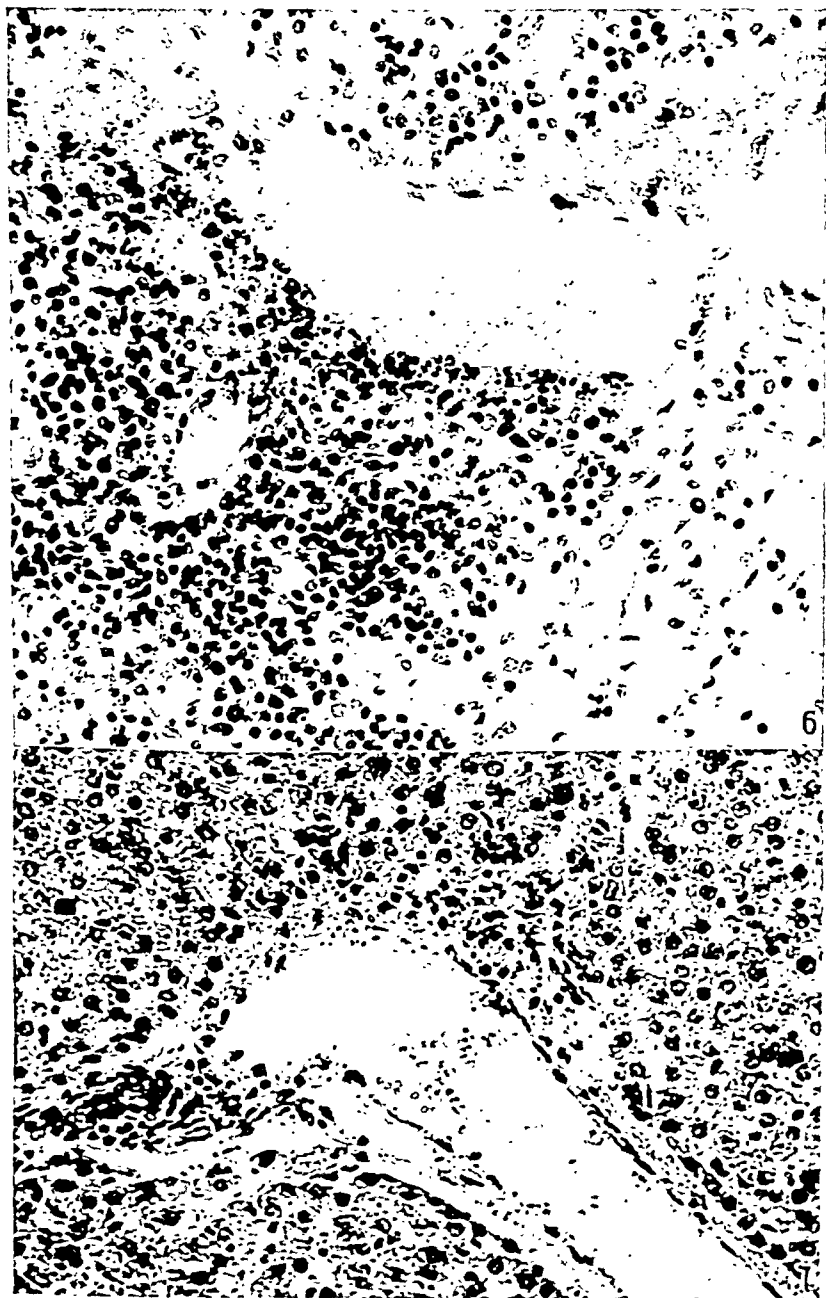
c











(Seifried and Cain: Histological studies on hog cholera. II)



## HISTOLOGICAL STUDIES ON HOG CHOLERA

## III. LESIONS IN THE VARIOUS ORGANS

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PLATES 23 TO 25

(Received for publication, April 22, 1932)

In the preceding paper we have described the changes in the blood vessels of swine infected with hog cholera virus and have stated that these lesions are the principal feature in the histopathology of this disease. In the present paper we propose to discuss in detail the histological changes in the lymph nodes, kidney, spleen, and liver.

*A. Blood-Forming Organs**1. Lymph Nodes*

The gross lesions in these organs are well known. Three types can be distinguished, (1) swelling and hyperemia, (2) hemorrhagic infiltration especially marked at the periphery, and (3) dense infiltration with red blood corpuscles, indicated by the dark red color of the lymph node parenchyma.

These three types of lesions are usually present in varying degrees in individual cases. In some cases the first type predominates, in others the second and third are more pronounced. Certain groups of lymph nodes are more severely affected than others. In our material there was a general lymphadenitis with the earliest and most characteristic changes in the cervical and thoracic lymph nodes. Although in general the edema and swelling are more frequent in early stages and the foci of necrosis and dark purple color of the lymph nodes usually appear in later ones, there is no rule in this respect. In very acute cases even the reverse may be true.

Papers published during the progress of our work indicated that there are various opinions with regard to the fundamental histological findings of the lymph nodes in hog cholera. In peracute cases of hog cholera Bel (1) saw a lymphatic

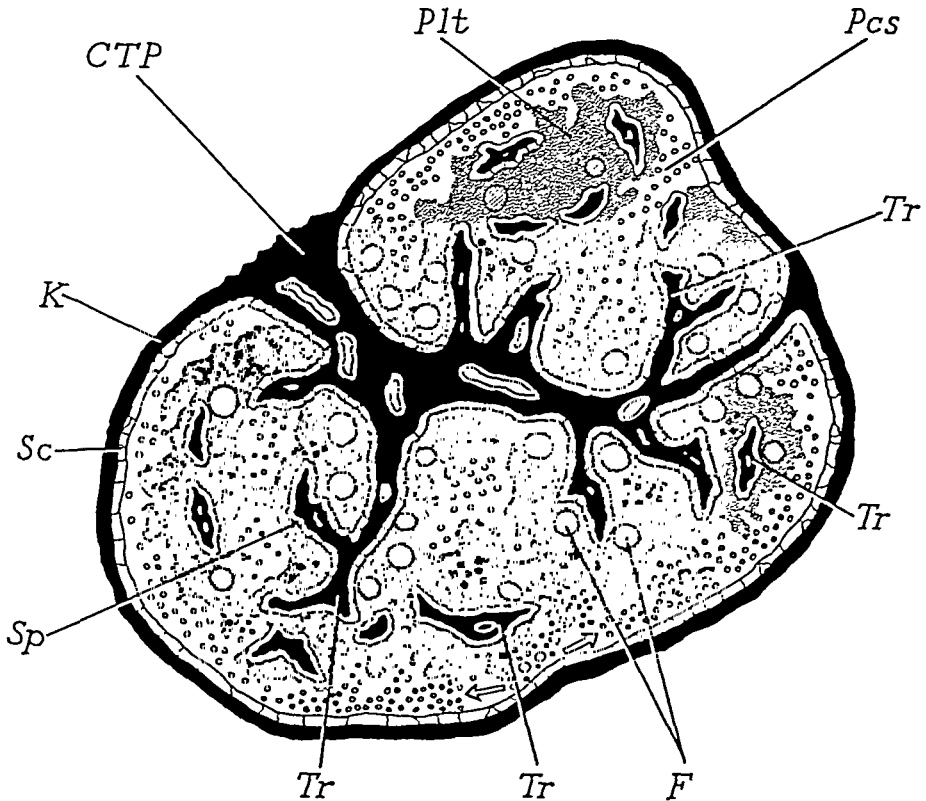
hyperplasia and hypertrophy of the follicles and hyperemia of capillaries from which he considered the hemorrhages in the parenchyma to be derived. In acute and subacute cases there was a pronounced hyperplasia in the lymph nodes which he thought resulted from a proliferation of small lymphocytes and macrophages. Nieberle (2) pointed out that, contrary to the common belief, acute cases of hog cholera are not associated with a hemorrhagic lymphadenitis. He holds the opinion that the red blood corpuscles, found in the lymph nodes, originate in other parts of the body and are carried by the lymph stream into the lymph nodes. Roehrer (3), on the contrary, concludes from the study of his material that the hemorrhages and necrotic foci in the various parts of the lymph nodes are due to lesions in the blood vessels.

*Normal Histology of Swine Lymph Nodes.*—For an understanding of the lymph nodes in hog cholera it is necessary to consider the normal histological structure. Details will be found in papers by Richter (4) and Trautmann (5); the principal points are brought out in the diagrammatic sketch (Text-fig. 1). The arrangement of the swine lymph node is almost the reverse of that found in other mammals. The lymphoid tissue with its follicles and germinal centers is situated centrally, while the cell-poor substance, which corresponds to the medulla in the lymph nodes of other mammals, is located peripherally but extends in the form of cords into the lymphoid tissue. This cell-poor substance consists of a syncytial network containing numerous capillaries. The connective tissue framework is represented by trabeculae that are rarely connected with the capsule. In the trabeculae are found blood and lymph vessels. There are two sinus systems, one around the trabeculae and the other under the capsule.

The afferent lymph vessels, according to Trautmann (5), enter the node at the connective tissue plate, run through the trabeculae, and open into the peritrabecular sinuses. The lymph only reaches the cortical sinus after passing through the cell-poor substance, except in those rare instances in which the trabeculae come into contact with the cortex.

*Lesions in the Lymph Node, Type 1.*—There are only slight lesions in the parenchyma. They consist mainly of a separation of cellular and fibrillar elements caused partly by edema, partly, as mitotic figures indicate, by a multiplication of reticulum cells. The edema is most pronounced in the cell-poor substance, especially that immediately surrounding the blood vessels. In a few cases fibrin can be demonstrated in small amounts within the edematous areas. Other changes, occurring irregularly, are: slight perivascular infiltrations in the cell-poor substance, perivascular necrosis of various degrees, swelling and degeneration of reticulum cells, increase of eosinophilic leucocytes, local hyperplasia of lymphoid tissue. Follicles and germinal centers are usually enlarged in size but decreased in number. Peritrabecular and cortical sinuses are entirely free from lesions, except for a slight endothelial desquamation, which may be normal. Trabeculae and capsule are edematous and in some areas show perivascular infiltration with lymphocytes and histiocytes. Perivascular hemorrhages and marked foci of necrosis are not seen in this stage.

*Lesions in the Lymph Node, Type 2.*—The most outstanding characteristic is the presence of hemorrhages in the cell-poor substance which usually lies in contact with the cortical and peritrabecular sinuses and is also found in cords within the lymphoid tissue (Text-fig. 1). In the latter tissue hemorrhages are not so pronounced. This peculiar distribution of the hemorrhages in the parenchyma



TEXT-FIG. 1. Diagram of a lymph node of swine. *Plt*, lymphoid tissue of the parenchyma; *Pcs*, cell-poor substance of the parenchyma; *Tr*, trabeculae; *F*, follicles; *Sp*, peritrabecular and *Sc*, cortical sinuses; *K*, capsule; *CTP*, connective tissue plate. The distribution of hemorrhages in the lymph node parenchyma in an early case of hog cholera is indicated by the small circles.

explains the marbled gross appearance of such nodes. In fresh hemorrhages the erythrocytes are well stained, while in older ones they show degenerative changes and appear as cell shadows. In larger hemorrhagic areas with dense accumulations of erythrocytes the structures are often indistinguishable. The frequent



perivascular arrangement of smaller hemorrhages around changed capillaries and smaller arteries indicates that erythrocytes must escape from these blood vessels. In some instances, however, these vessels show only swelling and proliferation of endothelial cells. Hemorrhages are seldom seen around larger blood vessels even when pronounced lesions are present in their walls. It is remarkable that red blood corpuscles are not found in the peritrabecular and cortical sinuses in these stages even when present in the cell-poor substance in great numbers. In only a few cases do they contain a limited number of erythrocytes either free or phagocytized in sinus endothelium or reticulum cells. In addition to these hemorrhages, more or less marked necrotic foci originating from changed blood vessels may be present in an individual lymph node. These foci may be small, but sometimes, by fusing together, they become so large as to occupy considerable areas of the parenchyma and may be so extensive as to be seen with the naked eye. The distribution of the necrotic areas resembles closely that of the hemorrhages. Only occasionally does the necrotic process affect the sinus walls; as a rule a narrow zone of normal tissue separates necrotic areas and sinuses (Fig. 1). Progressive necrosis involving trabeculae, capsule, and sinuses has been observed in only one case in which secondarily invading bacteria were present. No reactive segregation of necrotic tissue has been seen.

The changes of the remaining structures of the parenchyma correspond to those described in the lesions of Type 1. In the hemorrhagic areas there is a marked hemosiderosis and fatty degeneration of the reticulum cells, but not a striking increase in their number. In necrotic areas or in their immediate surroundings these cells likewise show fatty degeneration or are filled with phagocytized nuclear fragments. Focal hyperplasia of the lymphoid tissue is a rare finding; hyperplasia is found in the majority of cases. Follicles and germinal centers may be reduced in size and number or may be entirely lacking. The changes in the trabeculae and the capsule consist of edema, perivascular cell infiltration, slight hemorrhages, and small foci of necrosis associated with blood vessel lesions.

*Lesions in the Lymph Node, Type 3.*—Histologically these nodes show an advanced or final stage of the hemorrhagic infiltration found in the lesions of Type 2. Red blood corpuscles may be present in such numbers as to occupy almost the entire cell-poor substance as well as part of the lymphoid tissue. It is remarkable that even in such an advanced stage the peritrabecular and cortical sinuses may sometimes remain entirely free from red blood corpuscles. As a rule, however, the sinuses contain erythrocytes, often in such numbers as to distend the sinuses and rupture the reticular fibers. The presence of red blood corpuscles may cause atrophy of the lymphoid tissue to such an extent that only small islands remain.

These histological findings in the lymph nodes in hog cholera do not support Nieberle's (2) theory of resorption as a complete explanation for the lesions found in these structures.

We feel that the blood vessel lesions (see Paper II of this series) especially those found in the cell-poor substance in the lymph nodes, must be considered the source of edema, hemorrhages, and progressive necrosis in various parts of the lymph nodes.

These findings are in agreement with those recently described by Rochrer (3). An explanation is required for the fact that the hemorrhages are more pronounced in the cell-poor substance than in other parts of the parenchyma. The discovery of Trautmann (5) that the cell-poor substance is a special channel for the passage of the lymph between the peritrabecular and cortical sinuses offers a satisfactory explanation. As soon as they have left the changed capillaries in the cell-poor substance the erythrocytes are carried along with the lymph stream and are distributed in the manner described (Text-fig. 1). When present in great numbers they may enter the sinuses and lymphoid tissue.

## 2. Spleen

Out of thirty autopsies, the spleen showed no lesions in eleven cases and in seven it exhibited hyperplasia which was not different from hyperplasia in other infectious diseases. In the remaining twelve cases anemic infarcts were present.

Recently David and Schwarz (6) emphasized the diagnostic significance of these infarcts. Shortly before our investigations were concluded, Rochrer (7) described histological details of these lesions.

Macroscopically these infarcts appear as dark red, sharply outlined, irregularly shaped foci protruding above the cut surface of the organ. Sometimes they are a quarter of an inch or more in diameter and they are usually located in clusters on the margin of the spleen. On cross-section they are sharply demarcated from the normal tissue and appear in various forms, the most usual being a wedge shape, the apex of which is orientated toward the hilus (Fig. 2). In addition to these marginal foci there are, in a number of cases, smaller areas of various sizes in the parenchyma similar to the marginal infarcts and likewise surrounded by a hemorrhagic zone.

The histological examination of a great number of these infarcts indicates that they are based on the typical blood vessel lesions, described in Paper II of this series. The most pronounced lesions are in the follicular arteries, especially at the apex of the wedge-shaped infarcts (Figs. 2, 3). Frequently the swelling and hyalinization of blood vessel walls is so enormous as to lead to more or less complete occlusion of their lumen. In some cases thrombotic material is also attached

to the endothelium of such blood vessels. In other parts of the infarcts the blood vessels show exactly the same type of lesions. The parenchyma in their immediate neighborhood is found in various degrees of coagulation necrosis (Figs. 2, 3). In the early stages this necrotic process may be restricted to a single follicle but as a rule it extends to the entire infarct including pulpa, follicles, and trabeculae. Fibrin is missing. Hemosiderin is found in the changed blood vessel walls. Towards the hemorrhagic zone of the infarcts the necrosis of the parenchyma is less pronounced. In this area the reticulum contains numerous red blood corpuscles which form an intermediate zone between necrotic tissue and the peripheral hemorrhagic zone. However, this intermediate zone is sometimes entirely missing and in this case the necrotic tissue is immediately surrounded by a dense zone of erythrocytes which is most pronounced immediately beneath the capsule and which is often undergoing retrogressive changes. This explains the pronounced protrusion of the capsule in these areas as well as the destruction of the subcapsular parenchyma and sinuses.

The capsule itself shows in the early stages a slight thickening with a few hemorrhages while in later stages it may, in the infarct areas, become entirely necrotic.

The same infarcts are found in the parenchyma. Often only small microscopic necrotic foci are found. These extend from necrotic blood vessels, especially follicle arteries. In the parenchyma which is free from infarcts, and in spleens which show no gross lesions, slight blood vessel lesions of the type previously described can be demonstrated. They may be associated with perivascular hemorrhages or with slight perivascular necrosis. The most pronounced changes are: a more or less striking increase of pulpa cells, perifollicular hyperemia and hemorrhages of various degrees, infiltration of the Schweigger-Seidel's capillary tubes and trabeculae with red blood corpuscles, and striking aplasia of the spleen follicles.

To sum up it must be emphasized that the infarcts in the spleen in acute cases of hog cholera are caused by primary lesions of the follicular arteries leading to a more or less complete blocking of the circulation in the corresponding areas.

### *B. Urinary Organs*

Although the gross anatomical appearance of the urinary organs, particularly the kidneys, in typical cases of hog cholera is well known, detailed histological information concerning the lesions in these organs is lacking.

According to Proescher and coworkers (8, 9), the lesions in the kidneys correspond to an acute hemorrhagic glomerular nephritis, combined with a tubular nephritis. In peracute hog cholera cases, Bel (1) has described lesions of con-

gestive nephritis with interstitial hemorrhages, changes in the glomeruli and convoluted tubules, proliferation of the endothelial cells and infiltration with mononuclear phagocytes, and scarcity of polymorphonuclear leucocytes. In acute and subacute cases he observed inflammatory reactions in the glomeruli, degeneration of the uriniferous tubules, perivascular infiltration, degenerative processes in the epithelial cells, and cuffs of mononuclear cells around the vessels. Lüttschwager (10) found microscopical hemorrhages in cases that did not show typical gross lesions in the kidneys.

Macroscopically, petechial and ecchymotic hemorrhages are found quite regularly under the capsule and rarely in the medulla and pelvis. However, in some cases there are so few hemorrhages that diagnosis from macroscopic examination alone is difficult if not impossible.

The ureter, bladder, and urethra often show subserous hemorrhages or hemorrhages on their mucous surfaces, but the gross lesions in these organs are far less common than in the kidneys.

*Interstitial Tissue.*—The small hemorrhages scattered through the cortex and through other parts of the kidneys in practically all cases are regularly associated with the changes in the walls of the affected capillaries. However, the extent of the hemorrhages does not always correspond to the extent of blood vessel injuries. The fact that they frequently occur near the point of entrance of small blood vessels into renal glomeruli (Fig. 4) and that they are often limited to such a distribution in early cases where other changes are not recognizable, suggests that primary injuries occur in the walls of small vessels at these points. Small subcapsular accumulations of blood have a tendency to spread laterally under the capsule so that in cases accompanied by severe hemorrhages a number of these combine to form large subcapsular effusions of blood. Numerous small hemorrhages occurring in the cortex infiltrate interstitial tissue masses of scarcely greater dimensions than glomeruli. Sometimes they are extensive and greatly diffused. Dense accumulations of red blood corpuscles frequently cause the tubules to be widely separated (Fig. 4) and in some areas to be compressed or even collapsed. In more extreme cases the normal structures of the kidneys are obscured by the predominance of erythrocytes. Edema is imperceptible in most cases though it is occasionally present. When present it is more pronounced around affected blood vessels, mainly in the medulla and adjacent cortex.

Not only edema and hemorrhages but also perivascular infiltrations with macrophages (histiocytes) and lymphocytes are seen in the more advanced stages of the disease (Fig. 5). The infiltrating cells, frequently showing degenerative changes, sometimes become so numerous and widespread as to obscure other structures. Necrosis of the interstitial tissues is often continuous with that of blood vessel walls, but necrosis as a result of infarct formation is not found. Proliferation of interstitial connective tissues, occasionally seen, appears to be an extension of the process taking place in the adventitia of the blood vessels. In general the capsule of the kidney shows no significant changes.

*Renal Glomeruli.*—Capillaries in the renal glomeruli often show the same degenerative changes in their walls as do capillaries in other parts of the kidneys. Hemorrhages in the renal glomeruli occur frequently even in the early stages of the disease process (Fig. 4). Proliferation of the glomerular epithelium is seldom observed. In two cases the renal glomeruli and other structures showed amyloid degeneration. Bowman's capsules exhibit hemorrhages much less frequently than one would expect. Sometimes degenerative changes in the renal glomeruli extend to and include the walls of the corresponding Bowman's capsules. On the other hand, retrogressive changes of the cells lining Bowman's capsules are observed where there are no appreciable changes in the corresponding renal glomeruli.

*Tubules.*—The tubular epithelium shows varying retrogressive changes in a great many cases (Figs. 4, 5). In a few instances the tubules contain red blood corpuscles and fragments of corpuscles. These may have been caused by hemorrhages in the renal glomeruli or, in advanced stages of tubular degeneration, by the extension of interstitial hemorrhages into the tubules (Fig. 5). In some areas the degenerative lesions of the tubules are so pronounced that the structures are no longer distinguishable in the homogeneous necrotic masses.

*Ureters, Bladder, and Urethra.*—In the few cases examined the walls of the ureters, bladder, and urethra showed lesions of the blood vessels, perivascular hemorrhages, and accumulations of round cells similar to those observed in the kidneys and in the lymph nodes.

### C. Digestive Organs

#### 1. Liver

In twenty-three cases examined fifteen showed vascular lesions some of which were associated with hemorrhages. Although these hemorrhages were noticed especially around the capillaries the structure of the liver frequently makes it difficult to define the boundaries of hemorrhagic areas or to associate them with definite blood vessel lesions. In addition, the necrotic process in the blood vessel walls extends into the surrounding structures including the parenchymatous tissue and produces lesions very similar to those found in the other organs. In only one case was the necrosis found to extend from the central vein to include up to one-half of the affected lobule (Fig. 6).

In a number of cases infiltration of the interlobular connective tissue with mononuclear cells or connective tissue proliferation was observed, especially around certain bile ducts and blood vessels, as described by Eberbeck (11). Their occurrence is not constant enough to indicate the action of the hog cholera virus in this organ.

## *2. Intestinal Tract*

The intestinal tract was studied only in the early stages of the disease process and the same blood vessel lesions were found in the mucosa and, rarely, in the submucosa of the large intestine. Hemorrhages associated with them are as frequently observed as in other organs but foci of necrosis are seldom seen in the early stages of the disease. Hemorrhages are also found around blood vessels and capillaries that show no visible changes. Frequently the capillaries between the glands in the mucosa are enormously dilated and filled with red blood corpuscles. In addition to these lesions there are slight degenerative changes in the epithelial cells, slight edema in the connective tissue, and frequently an accumulation of round cells around the blood vessels in the mucosa, less frequently in the submucosa. In a few cases leucocytes are present in great numbers in the ducts of the glands. Necrosis which sometimes is present extends from changed blood vessels and seems to lead to the formation of ulcers. The study of typical larger ulcers in the intestinal tract is not included in this work.

## *D. Remaining Organs*

The remaining organs, notably the lungs, heart muscle, endocrine glands, and skin, were not included in routine examination because in general they are not the site of typical lesions in this disease. However, in a number of cases in which these organs were studied the same blood vessel lesions were found. Hemorrhages in the skin are not always associated with visible blood vessel lesions. Bronchopneumonic areas in the lungs were seldom present in our material.

## DISCUSSION

From this study it is evident that the blood vessel lesions described in the previous paper of this series are responsible in great part for the characteristic pathological picture of this disease. Not only the hemorrhages but also the foci of necrosis in the various organs and the anemic infarcts in the spleen find a logical explanation on this basis. The various organic lesions described in this paper are essentially the result of the more or less pronounced hemorrhages. Frequently inflammatory processes in various degrees, as found in the lymph nodes and kidneys, have complicated the histological picture. It must

be admitted, however, that frequently hemorrhages are found around blood vessels that do not show visible changes. On the other hand, even pronounced blood vessel lesions are not always associated with perivascular hemorrhages.

It is important to note that the duration of the disease in general does not determine the type of lesions found in the various organs. Often the course of the disease is so rapid that very severe lesions are found at earlier stages than one would expect. Roehrer (7) believes that the occurrence of splenic infarcts decreases with the length of the disease. However, this observation is not in accordance with our experience. No fundamental difference can be distinguished between the histological lesions in cases of hog cholera produced by virus alone and those complicated by secondarily invading bacteria. While we feel, in agreement with Roehrer (3, 7), that the number and the necrotic character of the lesions in the various organs are not dependent on the presence of secondarily invading bacteria, nevertheless, they are influenced to a certain degree by these organisms. This holds true especially for the lymph nodes, spleen, kidneys, and intestinal tract. In addition, the degree of degenerative changes, especially in the lymph nodes and kidneys, is evidently influenced by compression resulting from large accumulations of red blood corpuscles and round cells. Marked atrophy of the lymphoid tissue in the lymph nodes and spleen frequently seems to be due to the same cause. On the other hand the decrease of lymphoid cells and atrophy of follicles and germinal centers is often present in cases in which hemorrhages are not pronounced. These lesions in the lymph nodes and spleen seem to account for the lymphopenia which is almost always present in virus hog cholera. We are under the impression that the hog cholera virus is able to paralyze the lymphopoiesis in the early stages of the disease, hyperplasia of the lymph nodes and spleen being found very rarely and only in the early stages.

In connection with postmortem diagnosis, the following new observations in the histopathology of acute hog cholera demand consideration: first, the hog cholera encephalitis (see Paper I of this series), and second, the vascular lesions and the lesions in the various organs resulting from them. Waldmann, Roehrer, and Eberbeck (12) emphasize the presence of the encephalitis. We believe, however,

that the diagnostic value of hog cholera encephalitis is a limited one because it occurs in only 60 to 80 per cent of the cases and because it is still questionable whether it is strictly specific for this disease. As we have shown, there is the possibility of confusing hog cholera encephalitis and meningitis with the brain lesions occurring in swine influenza and with similar inflammatory processes of unknown nature as described by Ehrlich (13), Fekete (14), Doyle (15), Bendinger (16), and others. According to our present experience, the diagnosis of hog cholera cannot be based on the presence of an encephalitis alone. We feel that the vascular lesions and the changes resulting from them (especially in the lymph nodes, kidneys, and spleen) are far more important since they are almost invariably present. When present the splenic infarcts are of diagnostic value; but they are found in only 40 to 60 per cent of the cases.

In our work the histology in the various organs was very helpful in establishing a diagnosis in a number of cases in which the nature of the disease was doubtful from the gross anatomical lesions. Further investigation must show whether or not these changes can be relied upon in making a diagnosis of hog cholera.

#### SUMMARY AND CONCLUSIONS

1. The hemorrhages, foci of necrosis, and anemic infarcts met with in the various organs in virus hog cholera, result primarily from the vascular lesions described in a previous paper of this series. Although they are not dependent on the presence of secondarily invading bacteria, their severity is influenced by these organisms.

2. The lesions in the lymph nodes, spleen, kidneys, and central nervous system seem to be of special diagnostic value in questionable cases of hog cholera. The presence of an encephalitis alone does not justify the diagnosis of hog cholera because in the central nervous system of swine similar inflammatory lesions occur in other diseases.

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#### EXPLANATION OF PLATES

##### PLATE 23

FIG. 1. Necrotic blood vessels and large necrotic areas in the cell-poor substance of a lymph node. The sinus and peritrabecular tissue is not affected by the necrosis. Hematoxylin-eosin.  $\times 220$ .

FIG. 2. Necrotic marginal spleen infarct with peripheral hemorrhagic zone. Atrophy of lymphoid tissue in the other parts of the spleen. Hematoxylin-eosin.  $\times 12$ .

##### PLATE 24

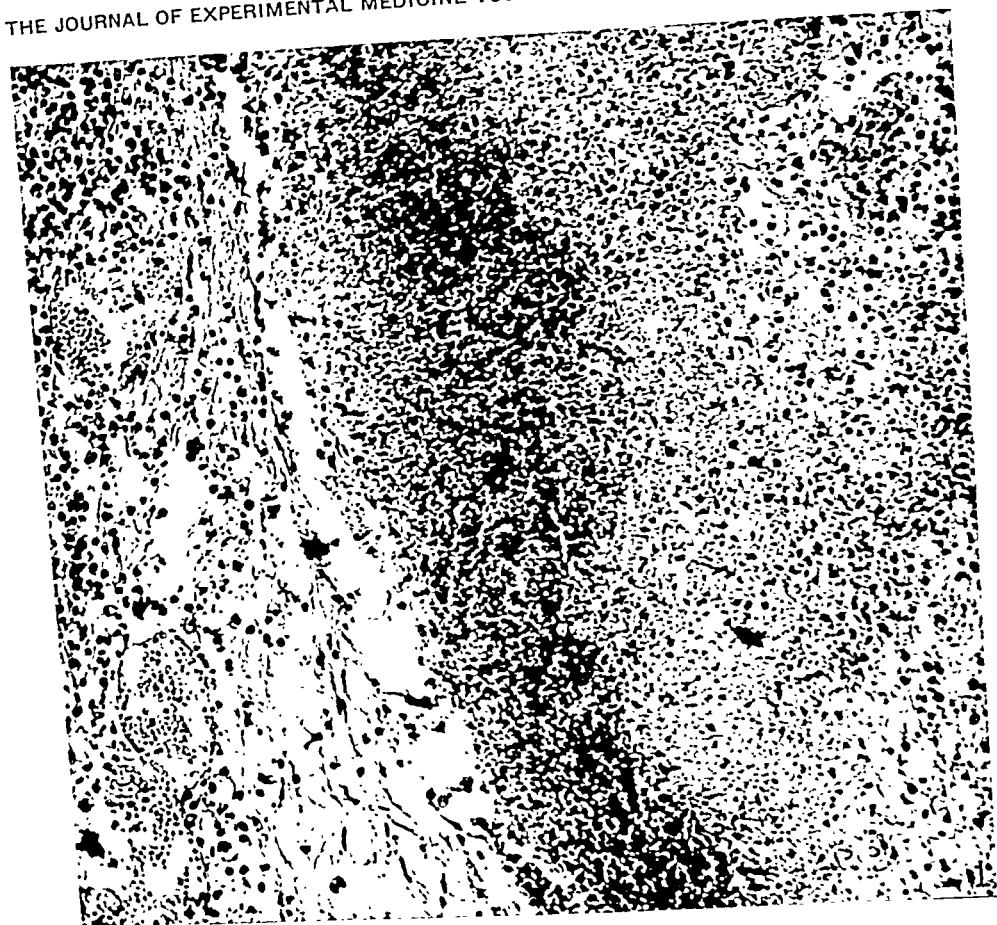
FIG. 3. Apex of necrotic spleen infarct with occluded lumen of a small artery. Hematoxylin-eosin.  $\times 245$ .

FIG. 4. Hemorrhages in a renal glomeruli and in the interstitial tissues, and degenerative changes of the tubules. Hematoxylin-eosin.  $\times 220$ .

##### PLATE 25

FIG. 5. Perivascular infiltration with macrophages and lymphocytes in the kidney. Hematoxylin-eosin.  $\times 260$ .

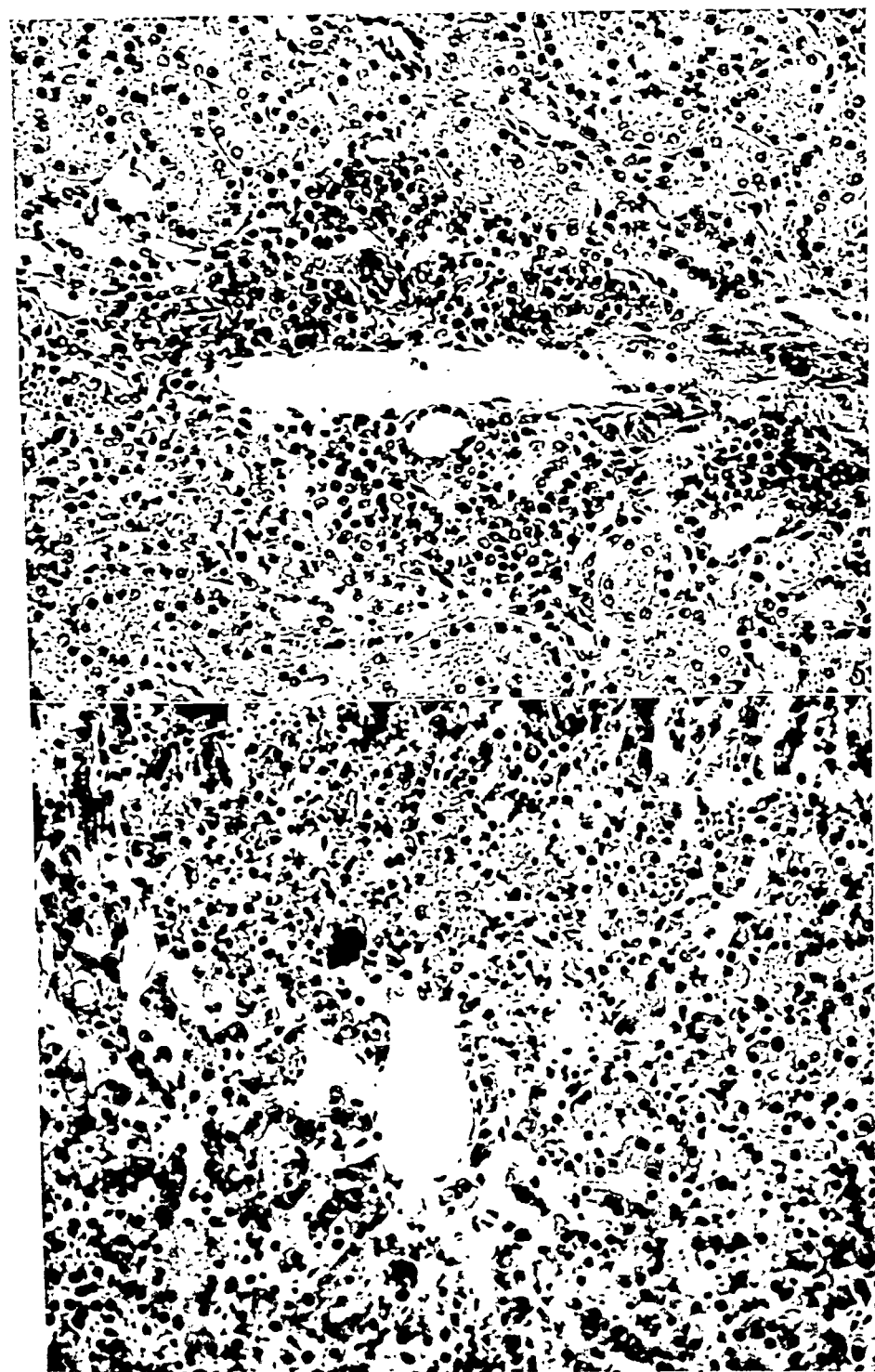
FIG. 6. Extensive necrosis in the neighborhood of a central vein in the liver. Hematoxylin-eosin.  $\times 300$ .













# LYMPH PRESSURES IN STERILE INFLAMMATION

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The literature contains but few reports on the normal pressure in peripheral lymphatics. In 1850, Noll (1), working in Ludwig's laboratory, found that the lateral pressure in the cervical lymph trunk of the dog, measured in terms of centimeters of a soda solution, varied between 8 and 18 mm. The lymph pressure rose during expiration and fell during inspiration, and was readily increased by any peripheral pressure such as stroking. In 1861, Weiss (2) made some measurements on the lymph pressure in the cervical lymphatics of dogs and colts, and found that in the case of the dog, the pressure varied between 5 and 20 mm. of a soda solution having a specific gravity of 1.080.

Measurements of the lymph pressure in the thoracic duct, of which there are several, have no bearing on our immediate problem, and those on the cervical lymphatics are so complicated by the depth and type of anesthesia and by respiratory changes that they cannot be considered typical of all peripheral lymphatics.

Starling (3), in 1894, remarked that if the foot of a dog was kept in water at 60°C. for 5 minutes, the lymph flow was increased and the lymph became much richer in protein. About a year ago two of the present authors (4) followed the changes in lymph protein and measured the amount of lymph produced under conditions of sterile inflammation. The extraordinarily large production of lymph invariably observed has led us to determine what pressures are reached in the lymphatics of the leg inflamed in a sterile manner.

## Technique

Dogs anesthetized intraperitoneally with nembutal (sodium-ethyl barbiturate) were used. The two main lymphatics at the ankle of the hind leg were isolated.



When size permitted, both vessels were cannulated and attached *via* a Y-tube to a vertical manometer. If one of the lymphatics was too small to cannulate, it was tied off. All other lymphatic paths were open. Injections of trypan blue into the legs of dogs confirm Baum (5) in showing two large lymphatics on the dorsal surface of the foot, in the region of the ankle, with a number of interconnections at different levels. There are also connections with smaller lymphatics draining the posterolateral side of the leg, so that in tying off these two lymphatics the drainage of lymph is diverted into collateral paths and is not materially obstructed. The pressure of lymph in the lymphatic was determined by allowing the lymph to flow directly into a calibrated vertical manometer.

In several of the experiments venous pressure measurements were taken at the same time. A cannula was tied into a small venous branch at the ankle in the same region which was exposed through the same incision as that in which the lymphatics were picked up. The venous cannula was attached to a citrate wash-out system and the pressure was recorded in terms of centimeters of water.

TABLE I

*Maximum Lymph Pressures in Sterile Inflammation*

No. of dog	Date	Weight	Treatment	Maximum lymph pressure
		<i>kg.</i>	<i>min. at 100°C.</i>	<i>cm. of lymph</i>
1	Dec. 8, 1931	23.0	2	106.4
2	Feb. 24, 1932	19.5	2	118.2
3	Feb. 26, 1932	20.0	2	78.0*
4	Mar. 2, 1932	22.0	2	118.8
5	Mar. 4, 1932	20.0	2	120.0

\* Graphite previously injected plugged cannula at one time.

After a series of normal readings had been taken, the whole paw up to the ankle was immersed either in water at 100°C. for a period of 2 minutes or in water at lower temperatures for varying lengths of time. Lymph pressure and venous pressure were recorded during the immersion and at intervals thereafter until the lymph pressure reached a maximum.

The rate and amount of lymph produced were determined in some experiments by the use of calibrated cannulas in the opposite leg which was treated in the same manner. Protein determinations were made refractometrically.

## RESULTS

In an anesthetized dog, the pressure in the lymphatics of the quiescent leg is not measurable. It has been our experience and the experience of others (Paschutin (6), Emminghaus (7) and Starling (3))

that no lymph is obtained from a cannulated leg lymphatic unless massage or passive motion is employed.

*Leg Lymph Pressure in Sterile Inflammation.*—Table I summarizes a series of experiments in which inflammation was produced by immersing the foot in water at  $100^{\circ}\text{C}$ . for 2 minutes. The maximum pressure obtained from a leg lymphatic was found to be 120 cm. of lymph and the range was from 78 to 120 cm. The lymph pressure rises immediately after the immersion and usually reaches a maximum in the course of 3 to 4 hours.

Since the lymphatics are well provided with valves, a possible fall in pressure after the maximum had been attained and held for some

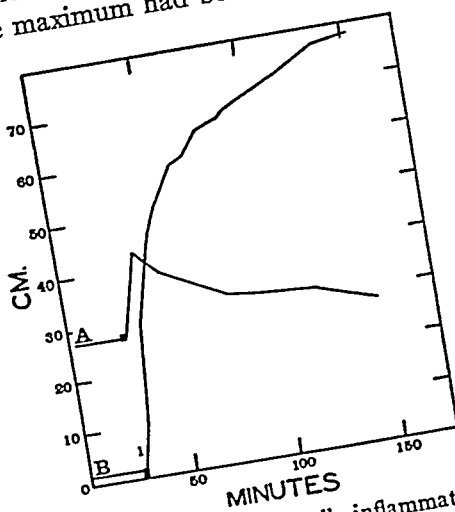


FIG. 1. Lymph and venous pressure in sterile inflammation. Ordinates, centimeters of water or lymph; abscissae, time in minutes. A, venous pressure; B, lymph pressure. At mark 1, foot immersed in water at  $100^{\circ}\text{C}$ . for 2 minutes.

time would not be recorded by the type of manometer used. To determine whether this was the case, in one experiment in which the maximum pressure had remained constant for over  $1\frac{1}{2}$  hours the manometer was disconnected and a second one was attached. In 20 minutes the pressure had risen 75 cm., and in 1 hour it had attained its previous maximum. In another experiment, after connecting with the second manometer the maximum pressure then attained was 20 cm. less, showing that in this case there had been a fall in the pressure. Fig. 1 illustrates a typical experiment in which both lymph and

venous pressure were recorded simultaneously. The venous pressure rises as soon as the water at 100°C. touches the foot and reaches a maximum about 16 cm. above normal in from 10 to 15 minutes. The lymph pressure rises rapidly, though always lagging a few minutes behind the rise in venous pressure. Shortly after the venous pressure begins to rise arterial pulsations are visible in the venous pressure manometer, indicating the direct transmission of arterial pressure through the dilated capillary bed to the venous side.

The protein content of normal leg lymph, which may vary from 0.5 to 1.5 per cent, was usually found to be around 4 per cent after a sterile inflammation had been produced. Under such conditions the capillaries become injured and extremely permeable to the blood proteins. After such an experience blisters were commonly found between the toes. In one experiment it was possible to measure the protein content of the lymph, of the blister fluid and of the tissue fluid obtained by inserting a needle into the subcutaneous tissue. Both the lymph and the tissue fluid had an average protein content of 3.5 per cent (three determinations, 15 minutes apart) while the blister fluid contained 2.5 per cent of protein.

Marked swelling of the leg occurs in from 7 to 45 minutes after the inflammation has been produced. No measurements were made of the amount of the swelling and its initial appearance was recorded in the gross. The increase in lymph production and the other lymph changes always occurred some time before swelling was noticed.

Since immersion of the foot in water at 100°C. produces a severe inflammation, it seemed worth while to see what effect simple hyperemia induced by water at lower temperatures would have on lymph pressure and to determine if possible at just what temperature changes in the lymph flow begin.

Fig. 2 illustrates a typical and complete experiment in which lymph pressure and venous pressure were recorded simultaneously in one leg, and in which the protein content and the amount of lymph produced were measured in the other leg which was treated in exactly the same manner. After a series of normal control determinations had been made, the two feet arranged side by side in identical positions were placed in a water bath in which the temperature could be raised to any desired degree. Control readings were also taken in water at

40°C. When the temperature was raised to 50°C., there was an average rise in venous pressure of 15 cm. of water but the effect on the lymph pressure and on the amount of lymph produced was extremely slight. Between 50° and 60°C., however, marked changes were pro-

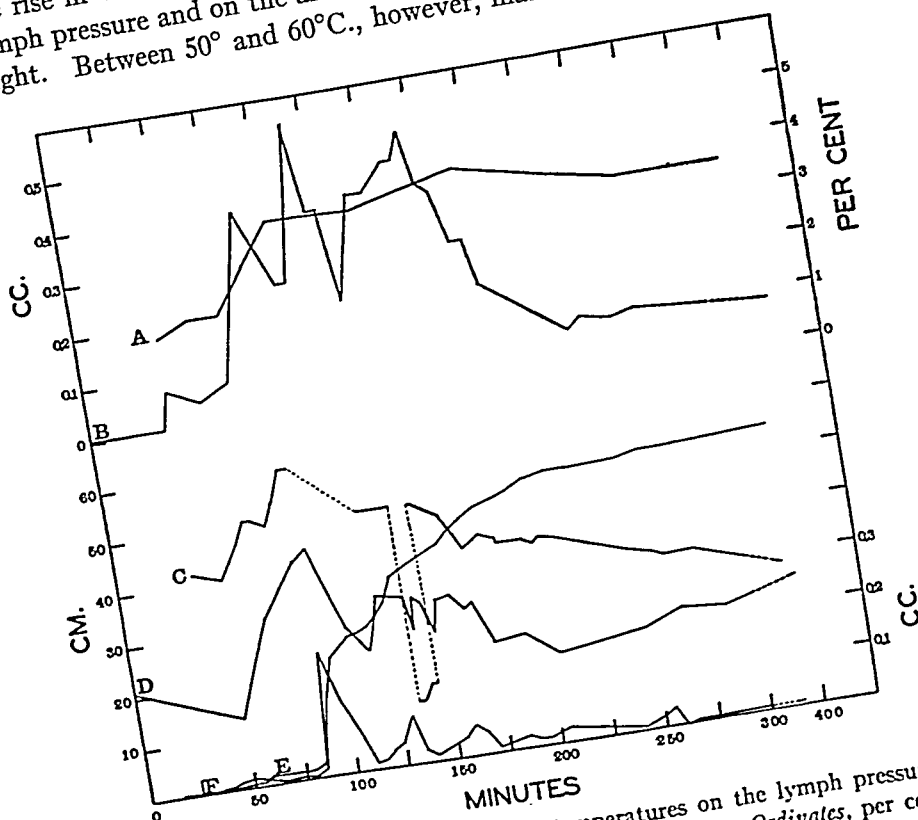


FIG. 2. The effect of water of varying temperatures on the lymph pressure, venous pressure and rate and amount of lymph produced. Ordinates, per cent of protein, cubic centimeters of lymph, degrees C. and centimeters of water or lymph; abscissae, time in minutes. A, per cent of protein in left leg lymph; B, amount and rate of lymph collected from left leg; C, temperature of water bath; D, venous pressure of right leg; E, lymph pressure of right leg; F, rate of lymph production in right leg.

duced. The venous pressure then rose 27 cm. above the normal average to a height of 44 cm. At this point also the lymph pressure began to rise and continued to rise thereafter. The amount of lymph pro-

duced by the opposite leg became very abundant and this lymph was more highly proteinized. The rate of lymph production of one leg plotted from the lymph pressure changes and measured directly on the opposite leg is seen to follow rather closely the changes in venous pressure produced by the water of varying temperatures, though there is always a slight lag.

In order to determine whether or not lymph obtained from legs inflamed in a sterile manner contains any toxic or depressor substance, intravenous injections into rabbits and cats whose arterial blood pressure was being recorded were tried. Normal lymph was used as a control. It was found that the inflammatory lymph contained no depressor substance and was as innocuous as normal lymph.

#### DISCUSSION

Under conditions of sterile inflammation such as have been produced here, there can be no question of generalized lymphatic thrombosis during the periods of observation, which were often 8 hours in length. The lymph flow is enormously increased and the ease with which the lymphatics may be entered can be demonstrated by injecting graphite into the subcutaneous tissue. Very often the injecting needle will enter a distended lymphatic directly and graphite will appear in the cannulated ankle lymphatic in less than a second after pressure has been applied to the plunger of the syringe. This is not the case with injections in the normal leg. One observation was made on the inflamed leg 24 hours after the onset of inflammation. In this case the lymph was still flowing though not as abundantly as it had been 12 hours previously.

The greatly increased extravascular fluid must cause the lymphatics to become dilated since the foot is able to swell freely in all directions. If the lymphatics were attached on their outsides to surrounding tissue, as Heimberger (8) has observed in the case of the capillaries at the base of the nail, then swelling would cause them to be pulled apart rather than to collapse, as the pathologists would have us believe, when fluid gathers in the extracellular spaces (Adami (9)). Our experience is quite in accord with some observations of the Clarks (10) on edematous tadpoles and chicks in which they found that in cases of generalized edema the lymphatics invariably enlarged and

that the delicate lymph capillaries did not collapse with the increased pressure outside the vessels, but on the contrary continued to absorb fluid until they became very much distended.

Menkin (11) has shown that trypan blue injected directly into the site of inflammation in the subcutaneous tissues is fixed in the inflamed area and fails to reach the regional lymph nodes. He has explained this failure of penetration by the occlusion of the lymphatic vessels and by the presence of a fine network of fibrin in the tissue spaces of the inflamed area. Because of the large amounts of free flowing lymph which we have always obtained up to 8 hours and in one case 24 hours after the onset of the inflammation, it is difficult to see how there could be any generalized thrombosis of the lymphatics in the region drained, yet trypan blue is fixed under such conditions. Examination of the subcutaneous tissues in such an inflamed leg shows that the tissue is extremely gelatinous in appearance. It would seem more likely, in the type of inflammation dealt with here, to explain the failure of trypan blue to reach the lymphatics on the basis of its inability to diffuse through such a mass, rather than by actual lymphatic blockage. In the dog in which the inflammation was of 24 hours duration and in which trypan blue had been fixed, mere handling of the leg caused the trypan blue to appear in the lymph in a very short time. In this case there could have been no lymphatic blockage.

#### SUMMARY

1. The normal lymph pressure in the legs of anesthetized dogs is not measurable.
2. The maximum pressure of lymph in the quiescent leg under conditions of sterile inflammation is around 120 cm. of lymph.
3. Venous pressure rises immediately in a region subjected to sterile inflammation and then slowly returns to normal. The rise in lymph pressure follows the rise in venous pressure.
4. Changes in lymph flow, lymph pressure, and protein concentration of the lymph occur when the part producing lymph is subjected to external temperatures between 50° and 60°C.

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# THE INFLUENCE OF THE PLASMA COLLOIDS ON THE GRADIENT OF CAPILLARY PERMEABILITY

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The wall of the capillary has long been considered on good evidence as behaving like an indifferent semipermeable membrane separating the blood from the tissues. All along the course of the little vessel the permeability has been supposedly the same, differences in the happenings at various points being referable to the interplay of several factors, diffusion, hydrostatic pressure, and osmosis chief among them. It has been held that in the first portion of the capillary the force of hydrostatic pressure dominates over the osmotic pressure of the blood colloids, with the result that fluid is forced out into the tissue to be reabsorbed again as the vein is approached, because the drawing power of the colloids there prevails. The recognition that the permeability of the capillaries of some organs—muscle, skin—increases progressively in the direction of flow (1) has necessitated a reconsideration of the rôle of the factors just referred to. Recent experiments have shown that the gradient of capillary permeability exists independently of the hydrostatic pressure (2). The present work was undertaken to disclose the relation to it of osmotic conditions and especially of the influence of the osmotic pressure of the blood colloids.

## Methods

The effect on the gradient of capillary permeability of greatly modifying the percentage of the circulating blood proteins has been directly followed. For this purpose several methods were employed.

Adult rabbits of about 2000 gm., fasted 24 hours but with access to water, were used throughout. They were anesthetized with sodium iso-amyl-ethyl barbiturate (sodium amytal—Eli Lilly and Co.) given intravenously, 0.5 to 0.7 cc. of 10 per cent solution per kilo. Freshly prepared solutions were always employed. For



surgical procedures light ether was required in addition. Loss of heat was prevented by keeping the animal on a warm pad.

Chicago blue 6B was the dye of choice, and voluntary muscle the tissue observed. As proven in a previous paper (1) specific affinities are not responsible for the staining, for the dye passes directly from the blood to the interstitial fluid, the initial coloration of the muscle being due solely to its presence in this fluid. It is poorly diffusible and the gradient of its escape is plainly visible in the gross color pattern. The initial escape of the dye, which is restricted to the further portion of the capillaries, can be followed with the microscope.

The warmed isotonic solution of purified Chicago blue (7 per cent in 0.45 per cent NaCl) was introduced in approximately 3 minutes; 7 minutes later the carotids were cut and the muscles examined at once. The general technique of examination has already been described, as also the color pattern indicative of the gradient of permeability along the muscle capillaries (1),—regularly distributed blue bars in long muscles, and a fern-like pattern in the sheet muscles of the abdomen, attesting to the progressive increase in the escape of dye along the further part of the capillaries. Variations in the rate and abundance of escape are frequent in normal animals; and hence only marked and constant departures from the characteristic findings can be deemed significant.

Some of the experimental procedures entailed important changes in the blood volume. The cell-plasma ratio furnished a rough index to them. To obtain this ratio duplicate blood specimens taken from the ear into standard 0.04 cc. pipettes were diluted with 2 cc. of 0.9 per cent NaCl in Hamburger tubes (3) and the proportion of cells determined after centrifugation. By this method changes in the cell volume due to alterations in the tonicity of the serum were avoided. In all calculations the normal blood volume of the rabbit was assumed to be 5.5 per cent of the body weight (4).

The carotid blood pressure was followed with a kymograph. Blood proteins were determined by the method of Van Slyke, using specimens taken from an ear vein. In certain cases the percentages of both albumin and globulin were determined in order to apply the osmotic pressure formula of Govaerts (5).

### *Effects of Increasing the Plasma Proteins*

The recent work of Adolph and Lepore (6) has shown that during the readjustment of blood bulk after introduction of hypo- and hypertonic solutions into the circulation, the voluntary muscles gain and lose fluid respectively. It is well known that the plasma constituents of transfused blood begin to leave the circulation almost at once, and it seems not improbable that some portion enters the muscle. If this is the case, the conditions affecting distribution of dye to this tissue during the readjustment after transfusion may be very different from those obtaining when the blood bulk has become stationary. For

this reason we have in some instances injected dye after the readjustment had been accomplished and in others while it was still in progress.

By the intravenous injection of large amounts of compatible heparinized plasma, or in some instances of serum, it proved possible to increase the concentration of the plasma proteins by as much as 40 per cent. This came about through a greater intravascular retention of protein than of the other plasma constituents. The procedure involved a considerable though transitory increase in blood volume. With the aim of avoiding this we worked out a method whereby the proteins of the blood could be concentrated *in vitro* for injection.

### *Concentration of the Blood Proteins*

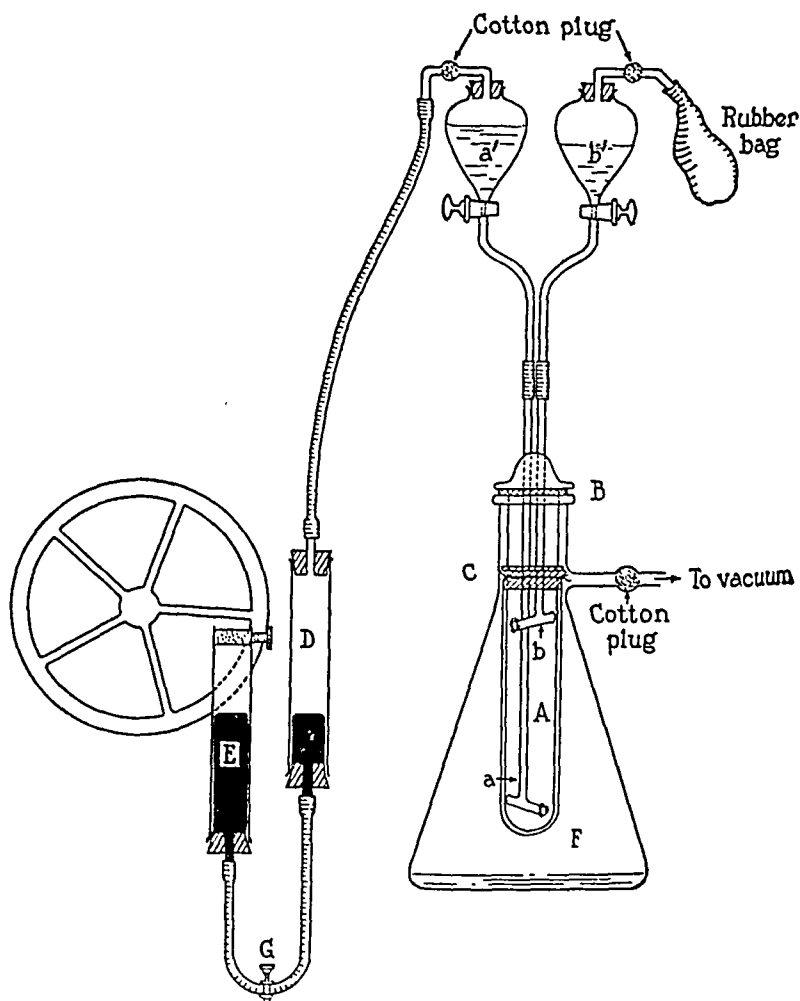
The fresh sterile sera of a number of rabbits are pooled and introduced into a specially devised concentrator.

*A* is a broad glass tube about 4 cm. in diameter and 18 cm. long, with one end rounded and the other expanded into a cap which rests at its edge on a ring of rubber (*B*), thus closing the filter flask (*F*) into which the tube is introduced. Two small tubes, *a* and *b*, pass down through the large one to end in cross-tubes which, extending through its wall, open near the top and bottom respectively. They terminate above in the bulbs *a'* and *b'*, the serum reservoirs. All the connections with *A* are sealed in.

A collodion tube slightly larger than the glass one, made according to Brown's (7) method, and just retaining proteins while letting crystalloids through freely ("80 per cent membrane"), is treated with 80 per cent alcohol and tied over *A* upon a rubber ring *C*. A close-fitting gauze jacket affords it support, preventing rupture when the pressure is lowered in the filter flask. This jacket does not appear in the diagram. The gauze has been previously sterilized and shrunk by boiling. About 2 mm. clearance should exist between the glass tube and the collodion surface, which latter has a filtering area of 250 sq. cm.

The apparatus is washed through by way of the serum reservoirs, first with 60 per cent alcohol, then with double distilled  $H_2O$ , and lastly with sterile 0.9 per cent NaCl solution. With the stop-cocks open, serum is now poured into one of the reservoirs, filling the space between the collodion tube and the glass tube *A* and mounting into the other reservoir. When both have been half-filled in this way, *a'* is connected with a pair of short vertical cylinders, *E* and *D*, containing mercury and joined by rubber tubing. The glass connection leading away from *a'* is sterile and is provided with a bulb packed with cotton to filter the air. Vertical excursion of the mercury columns in the cylinder *D* is provided for by attaching *E* to the circumference of a 10-inch wheel making 2 revolutions per minute, and the amount of the excursion is controlled by a screw-clamp, *G*. By this means pressure is brought to bear alternately on the serum in the reservoir *a'*, forcing it down into the space

between glass and collodion or sucking it up again. The excess displacement is taken care of by the reservoir *b'* which is connected with a sterile rubber bag to prevent free entry of the atmospheric air while permitting change of the serum level. The result is that the fluid in contact with the collodion surface is constantly changed. If now the pressure in the filter flask *F* is diminished to 400 mm.



TEXT-FIG. 1

Hg by means of the laboratory vacuum, filtration proceeds rapidly,—at a rate of about 4 cc. per minute to begin with, and somewhat more slowly as concentration progresses. The entire operation is carried out in an ice box at 2.5°C. In this way several hundred cubic centimeters of serum can be concentrated to about a fifth of its bulk within a few hours. The ultrafiltrate is sterile, colorless, protein-free and has the same freezing point as the original serum. The concentrate is

brownish red,—the ruddy tint being referable, as the spectroscope shows, to hemolysis imperceptible in the original serum,—but clear and not at all syrupy. The close agreement of the freezing points of ultrafiltrate and original serum, as well as the bulk of the separated fractions, attests the absence of any significant evaporation. This could be eliminated entirely by introducing into the flask a layer of sterile, washed paraffin oil, through which the ultrafiltrate would sink.

For determination of the protein concentrations in plasma and concentrate, Van Slyke's method was employed as has already been mentioned. Amounts of protein up to twice that contained in the animal's own total plasma were injected at body temperature into an ear vein of the rabbit used in the experiments. A test was made beforehand to find whether the concentrate hemolyzed or agglutinated the prospective recipient's blood. Hemolysis was never met and agglutination but rarely. In the latter case a compatible recipient was chosen. Since the serum had been outside the body more than 24 hours during the concentration there was no reason to expect the untoward effects recorded by Freund (8) for fresh serum; nor were they encountered. The injection was always well tolerated and the blood pressure remained high.

Hematocrit specimens taken after the injection of concentrate showed a diminution in the percentage of red cells much more considerable than could be accounted for by the fluid bulk introduced. It was plain that the increase in circulating protein had caused fluid to enter the blood from the tissues. The dye injection was not made until the blood volume had become approximately stationary. Hematocrit readings at this time indicated that the increase in volume was not great enough to reduce the protein percentage to that existing prior to the injection, and blood specimens taken just prior to dye injection proved that the concentration of protein per cc. of blood was indeed much increased, by as much as 40 per cent in individual instances, as already stated.

#### SPECIMEN PROTOCOLS

*Rabbit Injected with Normal Plasma.*—A male rabbit weighing 1200 gm. was fasted for 24 hours but allowed water. At 2:15 p.m. 0.7 cc. of 10 per cent amytal was given intravenously. Prompt hypnosis occurred, with a somewhat depressed respiratory rate. At 2:35 a first blood specimen was taken from the ear and at 2:50 the carotid was cannulated and the blood pressure tracing started. At 3:15, with the blood pressure at 70 mm. of Hg the injection of warmed heparinized plasma was begun. The first 20 cc. was given rapidly (in 2½ minutes) and the pulse rate slowed considerably, while the blood pressure rose. The injection was continued at a slower rate until 62 cc. in all had been introduced in 10

minutes. The blood pressure was now 80 mm. Hg. The animal was covered to conserve warmth and blood specimens were taken at intervals. The table shows the changes.

Time	Red cells	Serum protein	Blood pressure
<i>p.m.</i>	<i>per cent</i>	<i>gm. per cent</i>	<i>mm. Hg</i>
2:35	35	5.58	70
3:15	(62 cc. of plasma injected between 3:15 and 3:27)		
3:30	21	7.12	80
3:45	25		75
4:00	26		
4:30	27		73
5:00	27		
5:30	27		
6:00	27		65
6:30	26		Clotting in cannula
7:00	26		
7:30	26	7.58	

*Rabbit Injected with Serum Concentrate.*—A female rabbit weighing 1520 gm. was fasted for 24 hours. The red cell percentage was 43 and the serum protein 6.3 gm. per cent. At 1:40 p.m. 0.5 cc. of 10 per cent amyral was given intravenously. At 2:08 p.m. the carotid was cannulated; and at 2:21 the injection of warmed concentrated serum (24.7 gm. per cent protein) was begun. 12 cc. was injected in 4 minutes. An hematocrit reading taken 6 minutes later showed a red cell percentage of 34. At 2:50 the reading was 33, and at 3:00 again 34. The serum protein was now 8.4 gm. per cent.

In the animals of the foregoing protocols and in others treated in the same way, no dye was given. They were utilized merely to determine the course of the readjustment.

In the succeeding experiments dye was given at a time when the successive hematocrit readings indicated that the blood volume had become stationary. The amount of Chicago blue put into circulation was the same per cc. of plasma that yielded in normal rabbits a well marked coloration having the pattern indicative of the gradient of vascular permeability. Since staining intensity is conditional upon the concentration of dye in the plasma, allowance had to be made for the increase in plasma volume. This was calculated from the change in the red cell percentage, assuming that the original blood volume amounted to 5.5 per cent of the body weight. We were interested, not

in the eventual distribution of dye throughout the tissue of the rabbit, but only its first escape from the blood. Consequently the fact that the amount of stainable tissue was unaltered by the procedure could be disregarded.

The staining encountered in animals treated as described and killed after the routine period of 10 minutes from the beginning of the injection showed significant and constant deviations from the normal. The coloration was less intense than in normal rabbits and more narrowly localized to the furthest capillary region—the region of greatest permeability. One may liken the picture to that of the staining in a normal rabbit with one wash of color removed.

In the experiments just described conditions had become stabilized, the readjustment of the blood bulk having ceased. In the following experiments dye was given together with the concentrate and its escape occurred while the readjustment was going on. The change in the percentage of circulating protein could not be determined directly in these cases because the blood carried a nitrogen-containing dye.

A male rabbit weighing 1860 gm. was given amytal at 2:15 p.m. By 3:01 p.m. the carotid artery had been cannulated and connected with the manometer. At 3:10, 4.5 cc. of an isotonic watery solution of Chicago blue and 2.5 cc. of concentrate were injected in 4 minutes and 40 seconds. After another 20 seconds a further injection of concentrate alone was begun and 8 cc. more of it was introduced in 8 minutes and 30 seconds. The skin vessels showed moderate dilatation as the injection proceeded. The carotids were cut 2 minutes after it ended. Autopsy showed the familiar, barred, blue pattern in the voluntary muscles with the bands somewhat narrower and of a lighter hue than under ordinary circumstances.

As control a 1900 gm. rabbit was anesthetized lightly with ether and given 3.7 cc. of the same dye solution in 3 minutes. The carotids were cut after exactly the same lapse of time as in the experiment just described. Autopsy showed the usual, relatively pronounced staining.

In Table I the blood changes in the animals of the two groups of experiments are summarized.

### *Effects of Hypertonic Solutions*

To supplement the observations on the effects of increasing the plasma proteins, several experiments were made upon the course of the staining as influenced by the injection of a hypertonic solution of glu-

TABLE I  
The Blood Changes in Animals with Increased Plasma Proteins

Rabbit	A. Animals in which the blood bulk had become readjusted before dye was given																Percentage change in protein concentration		
	Body weight	Plasma volume	Amount of plasma or concentrate injected	Initial red cell percentage	Expected red cell percentage after injection	Actual red cell percentage stabilized	Elapsed period	Expected change in blood volume	Actual change in blood volume	Initial plasma protein concentration	Initial plasma protein	Amount of protein injected	Expected plasma protein concentration	Actual plasma protein concentration after injection	Elapsed period				
Injected with plasma	gm.	cc.	cc.	per cent	per cent	per cent	hrs., min.	per cent	per cent	gm. per cent	gm.	gm.	gm. per cent	gm. per cent	hrs., min.	per cent			
	P <sub>1</sub>	1300	39	65	45	23	33	4	5	+96	+36	7.7	3.0	4.7	6.6	7.9	4	5	+3
	P <sub>2</sub>	1200	42	62	36	19	26	4	5	+89	+38	5.58	2.3	4.2	6.6	7.58	4	5	+36
	P <sub>3</sub>	1440	51	72	36	19	33	4	5	+89	0	6.73	3.4	3.0	6.6	7.74	4	50	+15
	P <sub>4</sub>	1270	47	64	33	17	33	4	35	+94	+26	6.6	3.1	4.2	6.6	7.2	4	55	+9
	B	1520	48	12	43	38	34	30	30	+13	+15	6.25	3.0	3.0	10.0	8.38	30	30	+34
	C	1740	59	15	38	33	28	30	30	+15	+36	6.14	3.6	4.2	10.5	7.28	30	30	+19
B. Animals in which readjustment was taking place																			
Injected with serum concentrate	A	1860	64	11	37	33	29	3	3	+12	+28			2.8					
	D	1600	53	25	40	31	28	2	2	+29	+43			6.1					
	E	1680	52	14	44	38	34	3	3	+16	+29			3.5					

Infected concn-	P			10		
	1000	500	25	1000	500	25
1000	1000	1000	1000	1000	1000	1000
500	500	500	500	500	500	500
25	25	25	25	25	25	25

Infected concn-	P			10		
	1000	500	25	1000	500	25
1000	1000	1000	1000	1000	1000	1000
500	500	500	500	500	500	500
25	25	25	25	25	25	25

Infected concn-	P			10		
	1000	500	25	1000	500	25
1000	1000	1000	1000	1000	1000	1000
500	500	500	500	500	500	500
25	25	25	25	25	25	25



*Effects of Reducing the Plasma Proteins*

The percentage of circulating plasma proteins can be readily reduced by plasmapheresis. As ordinarily carried out this involves the repeated return of washed red cells suspended in Locke's solution. There is much in the literature to suggest that the best of salt solutions may harm the capillary endothelium, and it is essential to prevent such injury if one is to measure capillary permeability. In an initial series of experiments recourse was had to the ability of the animal to make up its own blood bulk after each removal of cells. Robertson and Bock have shown that forcing water by the alimentary tract after hemorrhage results in a remarkably rapid and effective restoration of the blood volume (9). Utilization was made of this phenomenon.

Warmed water or Ringer's solution was administered to amyralized rabbits by stomach tube, to the amount of 120 cc., and soon afterwards the anesthetized animal was bled repeatedly at short intervals and the cell sediment obtained on centrifugation was returned to the body by way of a femoral vein. To replace the loss of the first bleeding the cells from a compatible donor were used. Thereafter those of the host were injected, as obtained at each previous bleeding. The bleedings were carried out rapidly from a carotid artery into a 1 per cent heparin solution (1 cc. to 30 cc. of blood). The mixture was centrifuged and the plasma pipetted off as completely as possible. The cells were then suspended in 0.9 per cent NaCl, recentrifuged, the fluid discarded, and the sediment filtered through gauze before injection to remove the small amount of white granular fibrin which was always present in spite of the heparin. It was found that by the repeated bleedings and reinjections the plasma proteins could be reduced by about one-third in the course of 3 hours. The blood pressure tended to fall gradually but there was no other evidence of circulatory disturbance. The blood volume underwent a slight decrease as indicated by changes in the proportion of red cells in hematocrit specimens.

The amount of dye to be introduced was calculated on the basis of the plasma volume existing at the time of the injection. The blood pressure tracing was continued until the animal was killed.

In some further experiments the washed cells were made up to the original volume by the addition of ultrafiltrate obtained as a by-product to the concentration of protein by the method already described. Although the plasma was thus replaced by a fluid which lacked only the proteins of the blood, the blood pressure and volume fell to approximately the same extent as when no addition had been made to the cells prior to their reinjection.

Incidentally to the work, a number of tests were made upon rabbits of the merits of ultrafiltrate and of Ringer-Locke solution respectively, in restoring blood volume after hemorrhage. The carotid tracings did not indicate that the ultrafiltrate had any special advantages. Chicago blue 6B, given when the blood protein had been much reduced by plasmapheresis and the blood volume had become stationary, yielded a staining of the muscles notably greater than normal. This

TABLE II  
*The Blood Changes in Animals with Decreased Plasma Proteins*

yielded

TABLE II  
The Blood Changes in Animals with Decreased Plasma Proteins

Rabbit	Body weight		Plasma volume		Amount of plasma removed		Elapsed time		Initial red cell percentage		Final red cell percentage		Elapsed time after last removal of plasma		Percentage change in blood volume		Initial plasma protein concentration		Initial plasma protein		Approximate amount of protein removed		Final plasma protein concentration		Elapsed time after last removal of plasma		Percentage change in protein concentration	
	gm.	cc.	cc.	hrs., min.	per cent	per cent	hrs., min.	per cent	hrs., min.	per cent	hrs., min.	per cent	hrs., min.	per cent	per cent	gm. per cent	gm.	gm.	gm.	gm.	gm. per cent	gm.	gm. per cent	hrs., min.	per cent	hrs., min.	per cent	
L	1750	62	67	2 30	36	43																						
M	1920	65	97	3	38																							
N	1500	43	42	1 45	48	39																						
O	1900	65	60	2 37	38	39	1 20	42	1 42	42	43	1 42	20	42	2	2	5.65	3.3	2.6	2.8	3.58	2 55	45	2 55	45	2 55	45	
P	1830	58	56	2 42	42	43	2 55	42	2 55	42	43	2 55	42	43	2 55	42	6.11	3.8	2.9	3.76	2 45	45	2 45	45	2 45	45	2 45	45
Q	1830	62	54	3	38	39	2 45	42	2 45	42	43	2 45	42	43	2 45	42	5.82	3.4	2.9	3.16	2 45	45	2 45	45	2 45	45	2 45	45
R	1850	61	60	3	40	43	2 45	42	2 45	42	43	2 45	42	43	2 45	42	6.13	4.8	3.5	3.18	2 45	45	2 45	45	2 45	45	2 45	45
X	2300	80	75	2	37	40	1 10	40	1 10	40	46	1 10	40	46	1 10	40	5.8	3.9	3.3	3.63	1 10	10	1 10	10	1 10	10	1 10	10
T	2000	74	74	3	39	40	1 10	40	1 10	40	46	1 10	40	46	1 10	40	5.82	3.6	4.4	2 63	1 10	10	1 10	10	1 10	10	1 10	10
U	1850	61	105	3	40	46	2	40	2	40	46	2	40	46	2	40	5.8	3.7			3 0	4.04	3	4.04	3	4.04	3	4.04
V	1980	64	78	1 45	41	43	1 10	40	1 10	40	48	1 10	40	48	1 10	40	6.13											
W	1900	67	74	1 40	36																							

blood pressure had fallen somewhat  
indicative of the

happened although the systemic blood pressure had fallen somewhat as a result of the lessening in blood bulk. The bars indicative of the gradient of permeability were broader and more intense than ordinary, and in addition to them there was some generalized staining. In summary of the findings, one can say that the staining was like that in a vitally stained normal rabbit but with an extra wash of color everywhere superimposed.

Table II presents the blood findings in the animals treated as just described.

*The Influence of Adsorption of the Dyes*

The experiments prove that Chicago blue escapes more readily from the blood when its plasma proteins have been reduced, and with greater difficulty when they have been increased. One cannot conclude forthwith, however, that the differences are the result of a lessened or increased colloid osmotic pressure; for the possibility exists that they are due, in part at least, to adsorption of the dye on the plasma proteins. Marshall and Vickers have found that ultrafiltration of an aqueous solution of phenolsulfonphthalein through a collodion membrane yields a fluid of the same dye concentration as the original solution, but that ultrafiltration of a plasma mixture yields a more dilute filtrate (10). They suggest that certain phenomena in the renal elimination of phenolsulfonphthalein may be due to adsorption of the dye on the plasma proteins. Grollman showed that phenol red is indeed adsorbed on the blood proteins, more especially on the albumin fraction (11).

Tests for the fixation of Chicago blue on blood proteins by the method of ultrafiltration through collodion membranes are rendered difficult by the indiffusibility of the dye. Even through highly permeable membranes little passes from an aqueous solution in the course of several days. However, the diffusion method of Northrop and Anson in which a partition of porous glass is utilized (12), proved admirable for our purpose.

The cell, sealed with the porous glass, was filled with fluid containing a known amount of dye, and the stop-cock was closed, thus preventing alterations in the volume of the fluid by osmotic transport of water, which might have interfered with the free diffusion of the dye. An ultrafiltrate of rabbit serum, prepared in the way already described, was run into the outer jacket in sufficient quantity just to cover the porous disc. The fluids had been cooled previously to 2.5°C. and the apparatus was kept at this temperature.

The ultrafiltrate outside of the cell was drawn off and replaced at regular intervals of time and the amount of dye that had passed into it was estimated colorimetrically. This amount became constant when the concentration gradient of dye in the membrane had established itself, and this in turn was a function of the diffusibility of the dye and of the concentration within the cell of its freely diffusible portion, as distinct from that adsorbed on the blood proteins. The porous disc employed was 0.5 mm. thick and impermeable to proteins within the time limit of our experiments, as proven by preliminary tests specifically directed to the point.

## FREDERICK SMITH AND MACDONALD DICK

Equimolecular solutions of Chicago blue in ultrafiltrate and in rabbit serum were employed successively within the cell, and possible changes in the permeability of the membrane resulting from adsorption upon it of protein were controlled by a second series of determinations with dye in ultrafiltrate, carried out immediately after the cell had been emptied of serum, without washing it. In all instances the dye was present in the amount per cc. that had been introduced into the plasma of the rabbits used in our experiments.

The colorimetric determinations showed that the dye diffused more than three times as slowly from serum as from ultrafiltrate. Consequently the specimens of

TABLE III  
*Influence of Plasma Proteins on the Diffusion of Chicago Blue 6B*

	Dye in ultrafiltrate (15 min. specimens)	Dye in serum (hr. specimens)	Dye in ultrafiltrate (15 min. specimens)
Period of adjustment	25	41	37
	21		25
	18		21
	16	32	19.5
	15		16
Period of diffusion at constant rate			15
		25	
		21	
	14	19	14
	14		14
Relative amounts of dye escap- ing per hr. during constant period	14	19	14
	14	19	
	56	19	56

diffusate were removed every hour in the case of serum, and every 15 minutes in the case of the ultrafiltrate. Each specimen, as obtained, was made up to 25 cc. with Ringer's solution and compared in a Duboscq colorimeter with a 1:16,000 dilution of Chicago blue in Ringer's solution. During the readings the column of diffusate was kept constant (at 35) and that of the standard was varied. It follows that the figures procured are directly indicative of the relative concentration of dye in the various specimens. Thus for example the concentration of dye in a first specimen of ultrafiltrate, as recorded in the type instance of Table III, was nearly twice as great as in the last of the same series (25 as compared with 14).

It will be seen from Table III that dye diffused with far greater rapidity from the filtrate than from the serum. Since the amount of dye passing in unit time through the disc after the concentration gradient becomes uniform can be taken as a measure of the freely diffusible dye, it would appear that, in the instance given, as in others not here recorded, only about 30 per cent of the Chicago blue in the serum mixtures was available for diffusion, the remainder being adsorbed on the plasma proteins. The objection that the pores of the disc had become clogged with protein particles is disposed of by the control observations made after the use of a serum-dye mixture. The figures indicate that there was no mechanical clogging; indeed more fluid came through into the first control specimen than into the corresponding one of the preliminary series.

By a test of another sort the adsorption of Chicago blue was readily demonstrated. A marked reduction of the tinctorial value of phenol red is associated with its adsorption upon the blood colloids. The same holds true of Chicago blue, dilute solutions in serum being less intensely colored than similar ones in Ringer's fluid. To test whether this holds true when the dye is present in the amount per cc. of plasma employed in our animals, it has been necessary to utilize thin layers of fluid, since the deep color of the solutions tends to mask differences between them. Small drops of serum and of salt solution containing dye in the same appropriate quantity were placed side by side in a hemocytometer chamber having a double dais. Comparison showed a pronounced difference in their intensity of color, the mixture with salt solution being by far the deeper blue. No attempt was made to quantitate the difference.

#### DISCUSSION

The experiments show that when the percentage of the blood proteins is experimentally increased, the passage of Chicago blue from the muscle capillaries into the tissue takes place more slowly than usual and is confined in the beginning to the furthest portion of these vessels. When the proteins are decreased the dye escape is more abundant than usual and extends further back along the capillaries. In both cases the staining pattern is still indicative of an increasing permeability of the vessel wall as the venule is neared.

Are the altered osmotic conditions resulting from increase or decrease of the blood colloids responsible for these changes? Assuming this one might suppose that the passage of water from the tissue to the blood, consequent upon a sudden experimental increase in the blood proteins, would involve a flow in the opposite direction to that of dye escape, hindering the latter. But as a matter of fact, when dye is injected together with a hypertonic solution of glucose, and water is rapidly drawn into the circulation while dye passes out into the tissues, one finds no alteration in the distribution of dye to muscle or skin. The injection of dye concurrently with a plasma concentrate rich in blood proteins yields a staining quite as good as when the circulation has become stabilized after introduction of the concentrate, although water is doubtless passing from the muscle into the blood (6) while the coloration is going on.

If the osmotic pressure of the blood proteins does not condition the escape of dye, what can be the cause for the alterations encountered when the protein percentage has been changed? Our tests indicate that the amount of blood protein upon which the dye can become adsorbed is the effectual factor. Chicago blue when thus adsorbed is largely removed from the possibility of diffusion, as shown by our *in vitro* experiments; and the rate of staining with a dye is conditional not only upon its diffusibility, but upon the available amount of it (13, 1). The intensity of the staining of muscle with Chicago blue, the effective extent of the gradient of capillary permeability, and the presence or absence of diffuse coloration along the capillaries in addition to the local staining referable to the gradient, all have been found to vary with the amount of dye in circulation. The effective amount, in our experiments involving alterations in the per cent of plasma proteins, varied with this percentage, more or less being available accordingly as the percentage of circulating blood protein was decreased or increased.

It might be urged that adsorption upon tissues must also be reckoned with as complicating the findings; but Chicago blue is not immediately adsorbed in perceptible amounts upon the tissue of muscles into which it escapes (2). One has then only to consider adsorption upon the blood proteins, and this will account for the observed differences in the amount of dye escaping from the blood. The most striking evidence of

the influence of adsorption upon the proteins was obtained in the plasmapheresis experiments in which no fluid was added to the red cells restored to the circulation. In these experiments the carotid blood pressure fell, owing in part at least to a gradual lessening of the blood bulk. From previous knowledge it seems certain that there had been some compensatory restriction of the muscle circulation (14). Nevertheless the escape of dye from the capillaries was more abundant and the extent of the gradient of permeability was greater than under normal conditions, while, furthermore, dye escaped everywhere along the capillaries as does not happen ordinarily,—all this being due, it would appear, to the diminution in blood protein, which left more of the injected dye available for diffusion.

Can it be that the greater and greater escape of dye as the blood carries it along the capillary way, an increase essentially independent of the hydrostatic pressure (2) and of osmotic conditions, is caused by a progressive falling off in the amount of dye adsorbed upon the blood proteins as the blood flows along, with result that more becomes available progressively for escape through the capillary wall? Such a view entails the assumption that the changes taking place in the blood in its passage from arteriole to venule are such as would act to lessen adsorption. If this were the case, the gradient should vary directly with the magnitude of these changes, being less marked and even absent in proportion as they are prevented. Several facts negate this possibility. After section of the nerves to mammalian muscle the gradient persists although the blood undergoes but little alteration during its rapid passage through the dilated capillaries. The blood entering the capillaries of frog skin is essentially venous, having the lowest tension of oxygen and the highest of carbon dioxide to be found anywhere in the circulation, and it is rendered arterial on its way to the venules. Despite this reversal of the ordinary condition of affairs along capillaries, dyes escape in greater and greater amount from these vessels as the venules are neared, just as in the case of mammalian capillaries. Furthermore, when the circulation is cut off after dye has been allowed to enter the vessels of the mouse's ear, the staining that gradually ensues from capillaries dilated as the result of a previous ischemia shows that the gradient of dye distribution still exists, although now the stagnant blood has become venous everywhere. These facts would seem to rule

out the possibility that the progressive release of adsorbed dye is responsible for the increasing escape along the capillaries.

The fact that the gradient of capillary permeability exists independently of alterations in the percentage of the blood proteins is, of course, far from meaning that these are devoid of effect upon exchange between the blood and tissues. The edema of nephrosis and that produced experimentally by plasmapheresis (15) attest to an important influence of the proteins to hold water in the circulation as do some of the phenomena of the present experiments, notably the persisting increase in blood bulk after the injection of serum concentrate.

In theoretical considerations of the influence of osmotic pressure upon exchange through the capillary wall, the conditions have too often been oversimplified. The capillary is far from being completely impermeable to protein (16) and it lets through much more albumin than it does globulin. Osmotic pressure must always be referred to the particular membrane with which it is measured, for the osmotic forces of only those elements to which the membrane is impermeable are effective. If the collodion membrane utilized in osmotic pressure determinations held back all electrolytes, instead of the protein only, serum would give a value of 7 atmospheres instead of 40 mm. of Hg. As it is, collodion membranes impermeable to all the proteins are employed in tests for the osmotic pressure of serum, not membranes which let albumin through with relative ease; and these tests are made against an ultrafiltrate devoid of protein, not one containing it as lymph actually does. In consequence figures are obtained considerably higher than those to be expected had a capillary wall been utilized. The fact that this wall is not everywhere equally permeable, but becomes progressively more so as the venule is neared, complicates the state of affairs.

It is pertinent to inquire whether adsorption upon plasma proteins may not condition the distribution to the tissues of some normal substances as it does that of our dyes. That a large proportion of the bilirubin circulating in jaundice cases is adsorbed upon the proteins has recently been recognized (17).

In previous work the fact has been brought out that the escape of vital dye in progressively increasing amount along the capillaries is a phenomenon that occurs independently of alterations in hydrostatic



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In previous work the fact has been brought out that the escape of vital dye in progressively increasing amount along the capillaries is a phenomenon that occurs independently of alterations in hydrostatic

pressure, though its effective extent is influenced thereby. The same has now been found to hold true as concerns alterations in the percentage of plasma proteins. One must conclude, as from other evidence, that the gradient of vascular permeability disclosed by the escape of dye cannot be due to an interplay of hydrostatic and osmotic forces such as has generally been held to control exchange between the blood and tissues. Whatever the nature of the gradient,—and the evidence points to a structural cause for it,—it is a dominating factor in exchange.

#### SUMMARY

The influence of osmotic conditions on the gradient of capillary permeability disclosed by the distribution into mammalian muscle of vital dyes has been tested experimentally. The percentage of circulating blood proteins was increased in rabbits by the injection of compatible plasma, or of compatible serum concentrate obtained by means of a new method of ultrafiltration which has proved both rapid and effective. It was found that when this had been done and the circulatory conditions had stabilized themselves, the gradient of capillary permeability still existed, though its effective extent was less than under normal circumstances. When the percentage of circulating blood proteins was reduced, on the other hand, by repeated bleedings with return of the cells, either as such or suspended in the protein-free fluid obtained by dialysis of serum, the extent of the gradient of capillary permeability was broadened and dye passed out into the tissue more readily than usual from the capillary as a whole. In contrast to these findings injection of a very hypertonic dextrose solution during the period when dye was escaping had no perceptible effect on the gradient of capillary permeability.

The observed phenomena cannot be explained by a flow of dye-stained fluid into or out of the blood vessels. The gradient of capillary permeability exists independently of osmotic conditions, though its extent can be markedly influenced by altering the amount of circulating blood proteins. A considerable proportion of the dye used to study the gradient is adsorbed upon these proteins, as subsidiary experiments have shown. This happening provides a sufficient cause for the differences observed in the extent of the gradient when the percentage of proteins is increased or diminished.

The evidence like that of previous papers indicates that the cause for the gradient is to be found in a structural differentiation along the capillary, such that the barrier offered by its wall progressively diminishes on the way to the venule. Most current estimates of the effective osmotic pressure of the blood proteins fail to take into account the existence of local differences in permeability along the capillary.

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# INCIDENCE OF GASTRIC ULCER IN ALBINO RATS FED DIETS DEFICIENT IN VITAMIN B (B<sub>1</sub>)

By GILBERT DALLDORF, M.D., AND MINERVA KELLOGG

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PLATES 26 TO 28

(Received for publication, May 17, 1932)

The morbid changes associated with deficiency of the antineuritic factor of the vitamin B complex (hereinafter designated vitamin B) are not well established. This is in large part due to the fact that the vitamin B complex has been only recently separated into its component parts, the earlier anatomical studies having been made of animals which had been given diets deficient in more than one factor. With the perfection of experimental diets the prospects of understanding more satisfactorily the structural derangements occasioned by a deficiency of vitamin B seem brighter. The present report is one result of such a study. It is concerned with the occurrence of ulcerations in the gastric and duodenal mucosa of rats whose diet had been deficient in vitamin B.

## Method

64 animals have been studied. All were from the same stock, a strain of rats which has been used in the Department of Physiological Chemistry of Teachers College for many years. Most of the animals had been used by one of us (Kellogg) for various feeding experiments. The animals reported represent consecutive specimens derived from these experiments and no animals have been discarded. Until these experiments were commenced all of the rats had received the identical stock diet. All of them, during their course, were given the same basal ration (Sherman and Spohn's diet No. 107 (1)), which contains:

	per cent
Cascien (extracted) .....	18
Butter fat .....	8
Cod liver oil .....	2
Salt mixture .....	4
Corn starch .....	68



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This ration is deficient in the vitamin B complex and vitamin C. The thermostabile portion of the vitamin B complex (vitamin G) was supplied by adding to the ration large amounts of brewers' yeast which had been autoclaved for 6 hours at 15 pounds pressure. Vitamin C was not supplied because the rat does not require it (2). The animals were handled with great care, in a model animal room, and all of the groups were apparently free of infectious diseases throughout the course of the experiment.

At the termination of the experimental period the animals were killed with gas, the stomach immediately removed, slit open and placed in Zenker's solution. Our original purpose was to observe any evidence of gastric dilatation and intestinal stagnation which have been said to occur in vitamin B-deficient animals (3). Therefore in the first animals examined (Nos. 1, 3 and 11) only two levels of the gastric wall were sectioned. In all the other cases the stomach was cut in five segments and embedded in paraffin. Most of each block was sectioned, every third or fourth section being mounted and stained. More than 2,500 histologic preparations have been examined in the cases reported, each section including the entire circumference of the wall at one level. As routine the preparations were stained in Giemsa solution though other stains have been used for the special investigation of some lesions.

## RESULTS

*Gross Appearance of Stomach.*—Gastric dilatation was observed in only a few animals and it bore no relationship to the degree of deficiency of the diet. Camera lucida drawings of the histologic preparations were used for our comparisons. The results were so inconclusive they need not be given. Many of the completely deficient animals had small stomachs nearly empty of food. In some hair was present, generally in the rumen. Naked eye inspection showed no lesions of the stomach lining; and the ulcers which were found have never been positively identified in the gross specimens.

*The Incidence of Ulcers.*—Of the 64 animals examined, Nos. 1 to 29 inclusive had had little or no vitamin B in the daily ration, and, of these, twenty-one had one or more gastric ulcerations. Twenty animals (Nos. 45 to 64 inclusive) had larger amounts of vitamin B or were on a complete diet and in none of these were gastric lesions present. Nine animals (Nos. 36 to 44 inclusive) had been used for vitamin B assays. This involved a 40 day period of depletion followed by feeding repeated, increasing amounts of vitamin B sources. They

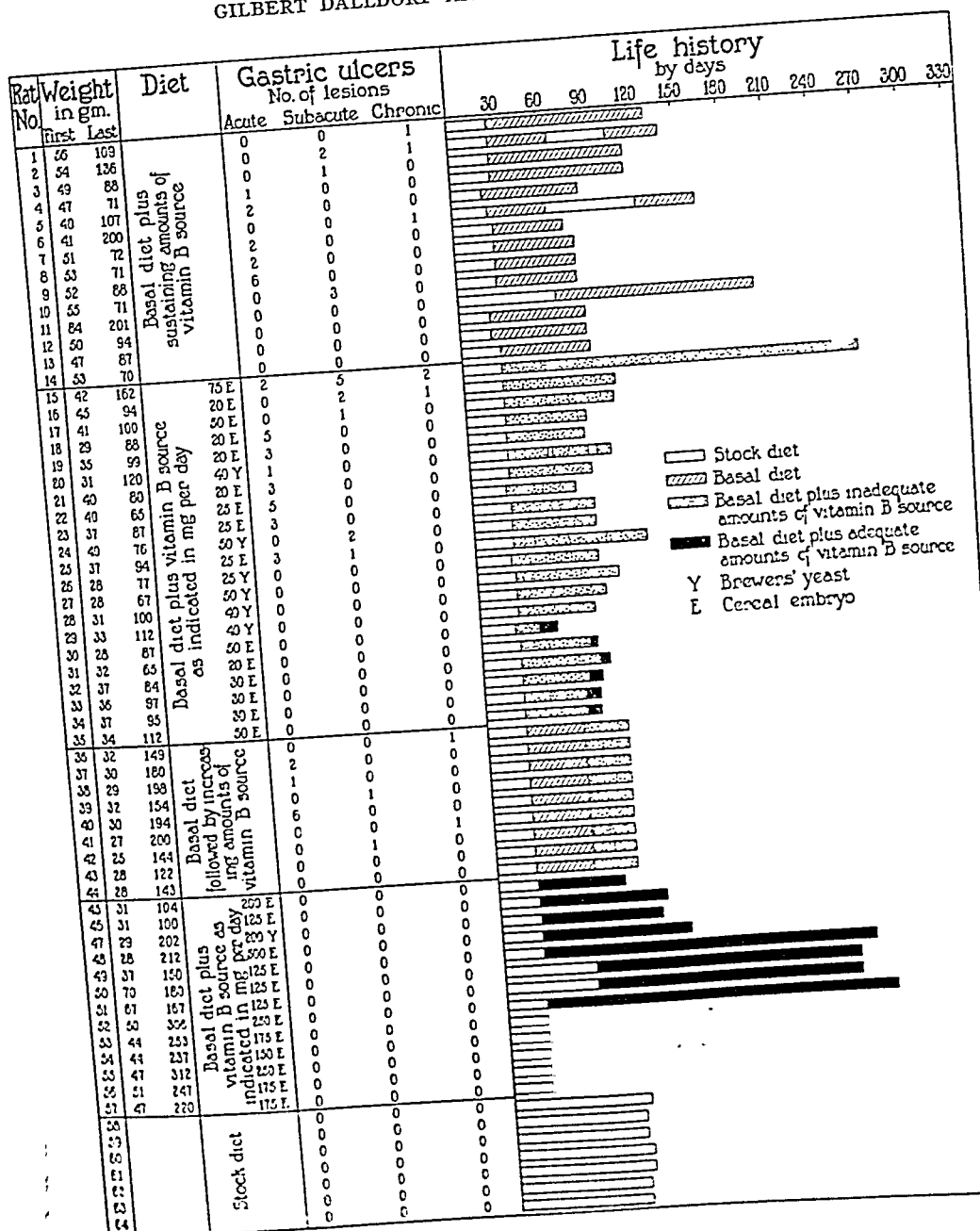


CHART 1

Vitamin B source used was cereal embryo ("Bemax," Schieffelin and Co., New York)

were comparable, in total vitamin B intake, to the first group. Seven of them had gastric lesions. A group of six animals (Nos. 30 to 35 inclusive) received only a small amount of vitamin B for periods of from 15 to 54 days. They were then given sufficient quantities of vitamin B source to relieve them of all clinical evidence of vitamin B deficiency. These animals are therefore not included among the twenty-nine truly deficient animals. In none of them were gastric ulcers found. The incidence of gastric ulcer in the deficient group of rats is 73 per cent, while in those animals protected by larger amounts of the identical vitamin B source no lesions occurred.

No difference in susceptibility to ulcer formation was noted between male and female animals.

Eight of the lesions were chronic, indurated ulcers or scars of the gastric wall. Forty-seven of the lesions were acute erosions and nineteen transitional forms showing some signs of induration and of reaction on the part of the gastric wall. The average duration of the cases in which chronic lesions were found was 114 days, while the average duration of the feeding period in those animals in which only acute lesions appear was 53 days. Acute ulcers were present with a chronic one in one stomach.

74 lesions were present in all, representing various stages in the development of a chronic ulcer strikingly similar to the chronic peptic ulcer of man, as well as forms that represent stages of healing. Therefore the histologic changes are a matter of considerable interest.

*Structure of the Lesions.*—The more acute lesions consist of minute punched out areas of necrosis of the gastric mucosa extending into the deeper half of this coat. The necrotic cells are often present in the plugs of debris lying within the crater, the cells and nuclei shrunken and compacted (Fig. 8). Frequently the cells of the necrotic mass and the base of the erosion have a glassy, acidophilic character (Fig. 12). In some of the cases a few polynuclear leucocytes are present. In many more these are absent. As the lesion becomes older it also enlarges considerably, the base becoming broader and slightly indurated. The basement membrane becomes involved, inflammatory cells are generally present and the necrotic mass may be lost. The outline of the lesion is still punched out, however (Figs. 5 and 6).

Later stages resemble chronic peptic ulcer with a dense scarred base overhung by adjoining gastric mucosa, sometimes hyperplastic and redundant (Figs. 1 to 4). The base of the ulcer shows digestion of its surface, contains leucocytes and often

thickened blood vessels and signs of vascular regeneration. Several of these lesions extend well into the muscular coats of the stomach. In such cases there is a pronounced inflammatory reaction in the submucosa about the lesion. Still older lesions have been observed in which the crater has become a dense collagenous scar, and one lesion shows a scar overgrown by a tuft of irregular and hyperplastic mucosa which arises from the margins of the lesion (Fig. 11).

*Distribution of the Lesions.*—The locations of the lesions are not known in all cases because they could not be seen in the gross specimen and often could not be definitely oriented in the histologic preparations. Most of them occurred along the lesser curvature or near it. This can be determined because the rat's stomach has an area of thickened mucosa along the greater curvature and this portion was affected in only two cases (both of which also had lesions elsewhere). Three of the ulcers occurred in the proximal duodenum.

As is shown in Chart 1 acute lesions were found in all stages and degrees of vitamin B deficiency while the chronic lesions were found only in cases in which the period of deficiency was long.

#### DISCUSSION

*Diet.*—The diet used contained neither vitamin B complex nor vitamin C. It is presumably ample in all other known constituents. The vitamin G factor was provided in each case by daily dose of 500 mg. of autoclaved yeast, but vitamin C was deficient in all of the animals. We have found no references pertaining to the gastric mucosa in rats on a scurvy-producing diet. In fact no structural effect from vitamin C deprivation has been demonstrated in the rat. This animal is, indeed, able to live and propagate in apparently normal fashion without antiscorbutic substance and to maintain, under conditions of complete deprivation of vitamin C, large stores of antiscorbutic in its tissues (4). A control group of thirteen animals received the identical basal ration augmented by vitamin B source, but no gastric ulcers were found in this group.

Three studies of rats deprived of vitamin B have been published which are of particular interest in the light of the present work.

McCarrison (5) found an occasional ulcer in the stomach and intestines of animals deprived of both vitamin B complex and vitamin C, and having a deliberately unbalanced basal ration. He concluded that a faulty diet predisposes

to gastrointestinal infection and therefore to a number of lesions of the tract among which is gastric ulceration. McCarrison did not attempt to associate the lesions found with one food factor alone and was apparently more interested in the other intestinal disorders, which in his series were more frequent. Findlay (6), some years later, studied a group of vitamin B-deficient rats but all of his animals were completely deprived of the vitamin and died after an average period of 32.5 days. He saw no gastric ulcers. A more recent study, by Sure, Thatcher and Walker (7), in which partially deficient as well as completely deficient animals were examined, more nearly resembles our own studies. They found a small gastric ulcer in one animal. They examined many different organs of the body. No importance was attached to the ulcer. None of these authors has described or illustrated gastric ulcers and a structural comparison with those found in our own rats is therefore impossible. It seems likely to us that other lesions might have been found in the animals of Sure, Thatcher and Walker if the search had been more inclusive. Since the lesions are so small that only a thorough histologic examination can be expected to identify them, it is not surprising that they may have been overlooked.

Since the lesions described are structurally similar to those which occur in man it becomes interesting to compare the pathology in the two species. The most widely held view of chronic peptic ulcer seems to be that it depends upon two factors, a preliminary acute lesion the result of a number of possible irritants, thrombosis, infection, etc., and a local or constitutional defect which precludes the healing of the acute lesion when once established. Local neurotrophic or circulatory abnormalities are frequently mentioned as local causes responsible for the non-healing of acute ulcers.

Our own experiments indicate that such is not the case in the rat for the present results can only be explained by the deficiency of one factor in the diet, and yet both acute erosions and chronic lesions appear to have been due to this defect. In other words, the dietary deficiency caused not only a tendency to erosion but likewise a tendency for certain of these acute lesions to become chronic.

The size of the lesions is similar to those which occur in man if adjustment is made for the differences in size of the organs involved. The size of acute lesions, as well as their location, is probably determined by the configuration of the mucosal folds. The exposed tips become eroded while those portions of the gastric mucosa protected by invaginations are spared. From our study of the ulcers in these rats the factor of gastric mucosal configuration as determining the site of the lesions is well substantiated.

## SUMMARY

73 per cent of a group of albino rats whose diet was deficient in vitamin B have been found to have ulcerations of the gastric mucosa. A control group was found to be free from gastric lesions.

Of 74 observed lesions eight were chronic, indurated ulcers resembling chronic peptic ulcer in man.

The chronicity of the ulcers seems to be related to the duration rather than the degree of the deficiency.

The lesions were generally located along the lesser curvature of the stomach, as is true in man.

The size of the lesions in rat and man are comparable if adjustment is made for differences in the sizes of the organs.

## CONCLUSION

Albino rats deprived of vitamin B commonly develop ulcers of the gastric mucosa.

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## EXPLANATION OF PLATES

## PLATE 26

FIG. 1. A low power photograph of a chronic gastric ulcer in which the inflammatory reaction extends through the muscularis mucosae. The crater was approximately 2 mm. in diameter.

FIG. 2. Higher magnification of the base of the same lesion.

FIG. 3. A chronic gastric ulcer which has invaginated and is overhung by intact mucosa. This lesion is similar to the chronic peptic ulcer found in man in its minute structure.

FIG. 4. Detail of the base of the same lesion. Note the thickened vessels and scarring.

## PLATE 27

FIGS. 5 and 6. Two small gastric ulcers with some induration of their bases but which have not extended through the mucosa.

FIG. 7. Two erosions of the gastric mucosa. The one on the left has scar tissue within it, the other is probably of more recent origin.

FIG. 8. A very small acute erosion with plug of necrotic cells in the crater.

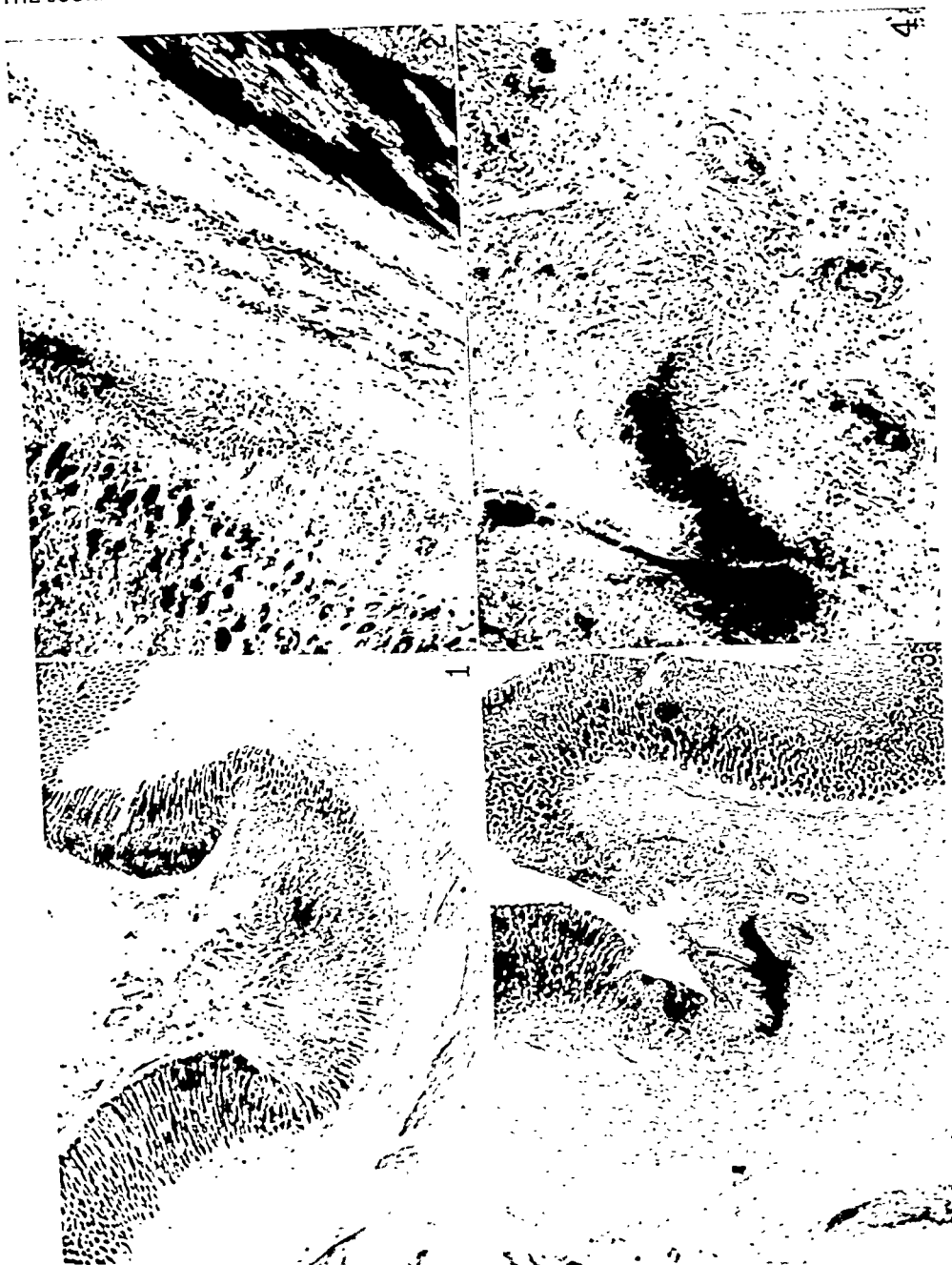
## PLATE 28

FIG. 9. A chronic ulcer of the proximal duodenum. The mucosa has closed over the defect. The lumen of the bowel may be seen at 11 o'clock. The ulcer is scarred, shows vascular lesions. Its base contains many polynuclear leucocytes.

FIG. 10. A large acute erosion in which the cellular necrosis is extending downward and towards the left. Several areas of a glassy, acidophilic appearance are present in the center.

FIG. 11. An area of gastric mucosa interrupted by a scar and overgrown by atypical mucosa which arises from the margins of the scar. This was found in Animal 6 and is assumed to be a healed ulcer. It is not listed in Chart 1.

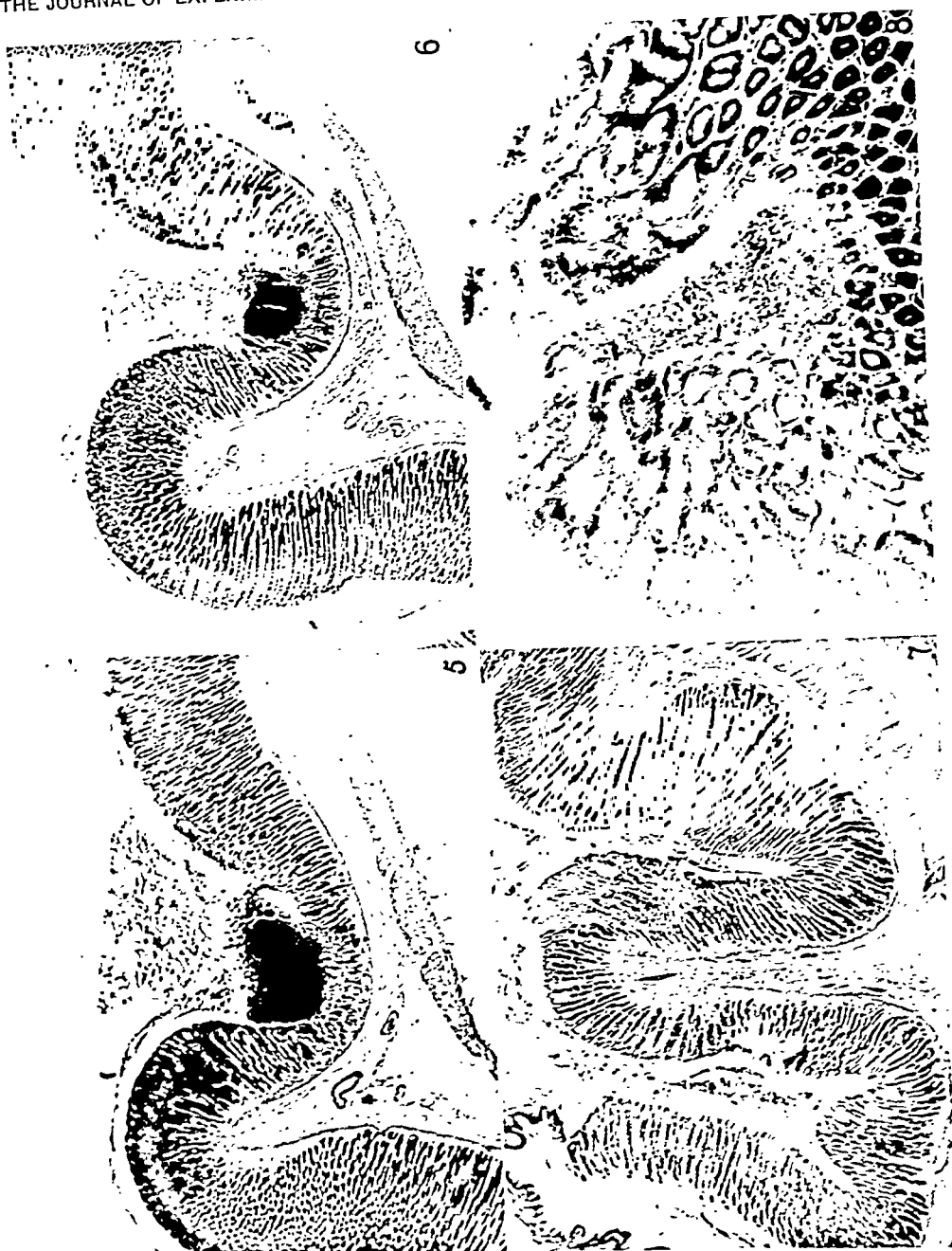
FIG. 12. An acute erosion of small size but containing leucocytes.



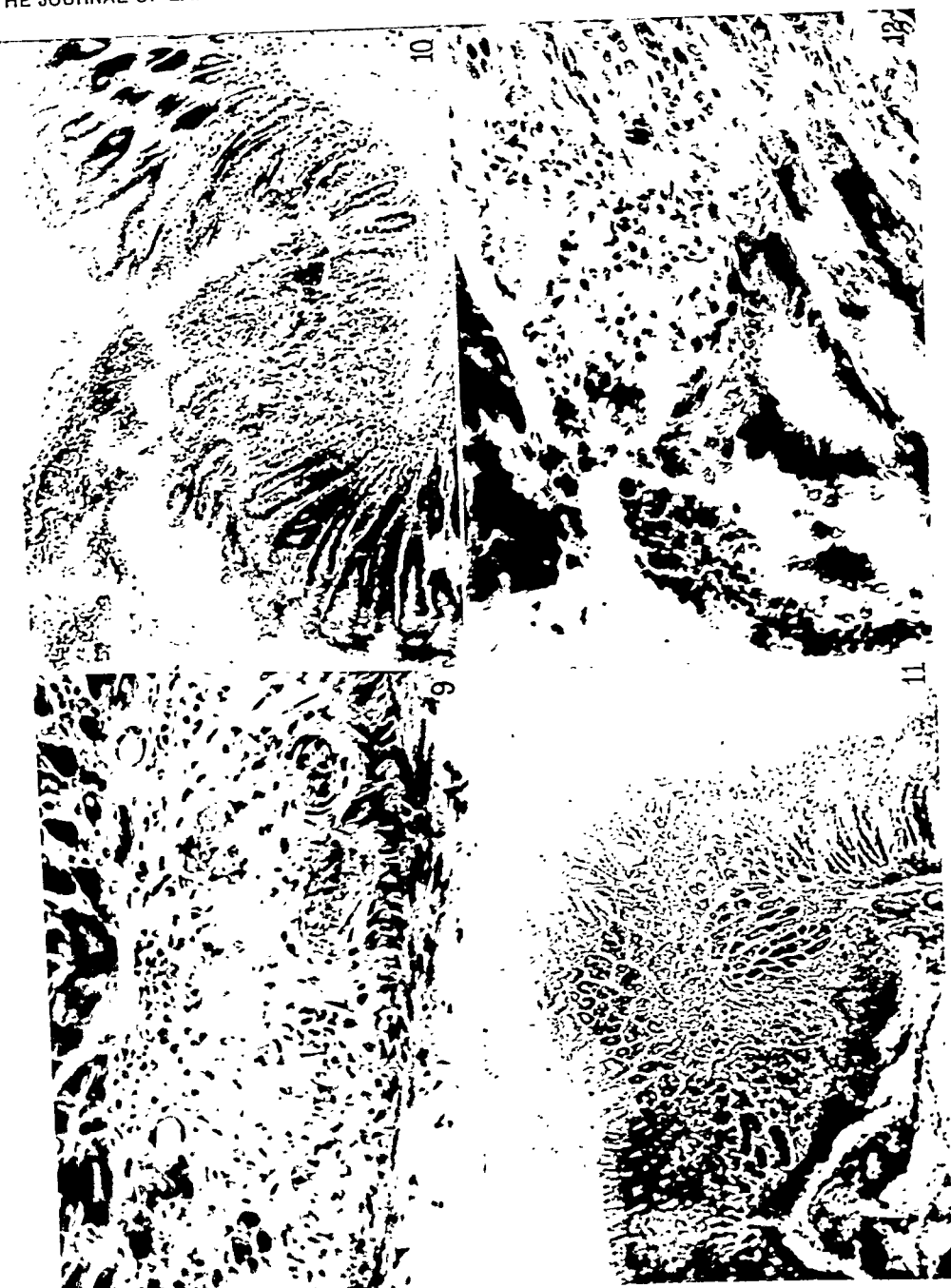
(Dall'Inda and Kellomäki: Gastric ulcer in rats lacking vitamin B<sub>12</sub>)





(Dall and Kellner. Gastric ulcer in rats lacking vitamin B<sub>12</sub>)





(Dall and Rolley: Gastric ulcer in rats lacking vitamin B)



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# SEROLOGICAL REACTIONS WITH SIMPLE CHEMICAL COMPOUNDS (PRECIPITIN REACTIONS)

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Serological reactions with chemical compounds of simple constitution were demonstrated for the first time by means of the so called inhibition reaction (1, 2). This subject has been discussed (1), and the arguments given for the view that the inhibition phenomena are caused by a combination of the inhibiting substances with antibodies, thereby preventing their precipitating action. Although there is no reasonable doubt as to the validity of this explanation which is strongly supported by the close similarity in the specificity of inhibition and precipitin reactions and the analogy to the inhibition produced by an excess of precipitable substances, it has remained of value to furnish direct experimental proof of the combination of antibodies with low molecular compounds by a reaction which does not involve the use of protein antigens.

In experiments on anaphylaxis to azoproteins it was shown that previous injection with azodyes protected sensitized guinea pigs against the shocking action of azoproteins containing the same azo component (3-5). Since this protection was still evident after the dyes had practically disappeared from the circulation, the effect was attributed to a neutralization of antibodies. In some cases the injection of the dyes was even followed by typical anaphylactic symptoms. Klopstock and Selter (6) reported that they obtained complement fixation reactions with immune sera to azoproteins, and emulsions of lecithin preparations to which had been added diazotized *p*-arsanilic acid or metanilic acid. Interesting experiments were carried out by Marrack and Smith (7) who were able to show that the diffusion of an azodye prepared from *p*-arsanilic acid is interfered with by the addition of an immune serum specific for the azo component.

Attempts made in the course of studies on azoproteins to produce specific precipitin reactions with simple azodyes did not lead to consistent results. Recently, a special group of azodyes was found with

which such reactions could easily be demonstrated. These substances were made from a series of aminoanilic acids, with aliphatic side chains containing from two to eight carbon atoms, by diazotization and coupling to resorcinol or tyrosine. For the production of the immune sera, antigens were prepared by diazotizing the same aminoanilic acids and coupling the diazo compounds to protein.

### *Technique*

*Preparation of Nitroanilic Acids.*—The method used was essentially the same as that described in a previous paper (8).<sup>1</sup> A finely ground mixture of equimolecular quantities of dicarboxylic acids (malonic, succinic, glutaric, adipic, pimelic, and suberic acids), and para-nitroaniline was melted in a paraffin bath at 170–175°C., and the mixture was stirred continuously for a period of 45 minutes. In the preparation of nitromalonic acid, the temperature of the bath was kept at only 130–135°C. In all cases except that of succinic acid it was found necessary to use fused anhydrous zinc chloride in the condensation in order to obtain satisfactory yields.

The powdered zinc chloride (8 gm. for 13.8 gm. of *p*-nitroaniline) was added to the melt in 5 portions during the first 15 minutes. After heating for 45 minutes, the mass was poured into about 10 volumes of water in a porcelain dish and heated on the steam bath until broken up. During this process, concentrated sodium hydroxide was added in small amounts until the solution remained alkaline to litmus. After filtration, the insoluble material was finely ground and extracted a second time with hot water and alkali in the same manner. The aqueous extracts were combined, made neutral to litmus, and after standing in the ice box overnight, the filtered solution was made acid to Congo red by addition of concentrated hydrochloric acid. The precipitate was washed with water and dried. Of para-nitromalonanilic acid only 20 per cent of the theoretical yield was obtained due to decomposition of malonic acid during the fusion. In the other cases the yield was 40 to 50 per cent.

The following nitroanilic acids were prepared.

Para-nitromalonanilic acid ( $\text{NO}_2\text{-C}_6\text{H}_4\text{-NH-CO-CH}_2\text{-COOH}$ ): Crystallized from boiling 25 per cent alcohol. Microscopic platelets. Melting point: 157°C., with gas formation.

Titration: 0.1279 gm., dissolved in 80 per cent alcohol, required for neutralization 5.7 cc. *N*/10 NaOH. Formula  $\text{C}_9\text{H}_8\text{O}_5\text{N}_2$  requires 5.7 cc.

Para-nitrosuccinanilic acid ( $\text{NO}_2\text{-C}_6\text{H}_4\text{-NH-CO-CH}_2\text{-CH}_2\text{-COOH}$ ): Crystallized from 25 parts of boiling 25 per cent alcohol. Long, microscopic, prismatic platelets. Melting point: 194–195°C.

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<sup>1</sup> *p*-Nitrooxanilic acid was made according to the method described by Ossian Aschan (9).

Titration: 0.119 gm., dissolved in 80 per cent alcohol, required for neutralization 5 cc. N/10 NaOH. Formula  $C_{10}H_{10}O_5N_2$  requires 4.96 cc.

Para-nitroglutaranilic acid ( $NO_2-C_6H_4-NH-CO-CH_2-CH_2-CH_2-COOH$ ): Crystallized from 40 parts of boiling 25 per cent alcohol. Long, thin, microscopic crystals. Melting point: 170–171°C.

Titration: 0.2199 gm., dissolved in 80 per cent alcohol, required for neutralization 8.7 cc. N/10 NaOH. Formula  $C_{11}H_{12}O_5N_2$  requires 8.72 cc.

Para-nitroadipanic acid ( $NO_2-C_6H_4-NH-CO-CH_2-CH_2-CH_2-COOH$ ): Crystallized from 25 parts of boiling 50 per cent alcohol. Narrow microscopic platelets. Melting point: 174–175°C.

Titration: 0.133 gm., dissolved in 80 per cent alcohol, required for neutralization 5 cc. N/10 NaOH. Formula  $C_{12}H_{14}O_5N_2$  requires 5 cc.

Para-nitropimelanilic acid ( $NO_2-C_6H_4-NH-CO-CH_2-CH_2-CH_2-CH_2-COOH$ ): Crystallized from 50 parts of boiling 25 per cent alcohol. Long, narrow, microscopic platelets. Melting point: 147–148°C.

Titration: 0.1530 gm., dissolved in 80 per cent alcohol, required for neutralization 5.45 cc. N/10 NaOH. Formula  $C_{13}H_{16}O_5N_2$  requires 5.46 cc.

Para-nitrosuberanilic acid ( $NO_2-C_6H_4-NH-CO-CH_2-CH_2-CH_2-CH_2-CH_2-COOH$ ): The crude substance often contained some suberic acid from which it was easily freed by redissolving in water with sodium hydroxide and partial precipitation from this solution with hydrochloric acid which caused the nitro compound to separate first. It was then dissolved in boiling 50 per cent alcohol and crystallized upon cooling of the hot solution as microscopic platelets. Melting point: 158–159°C.

Titration: 0.147 gm., dissolved in 80 per cent alcohol, required for neutralization 5.05 cc. N/10 NaOH. Formula  $C_{14}H_{18}O_5N_2$  requires 5 cc.

*Preparation of Aminoanilic Acids.*—The nitroanilic acid, dissolved in about 3 parts of water by addition of a slight excess of ammonium hydroxide and heating, if necessary, was added to a hot solution of ferrous sulfate, 7 aq. (6.5 mols for each mol of nitroanilic acid) in 2.5 parts of water. A 28 per cent ammonia solution (10 cc. for each 12 gm. of ferrous sulfate, 7 aq.) was added in 5 equal portions over a period of 10 minutes, shaking well with each addition. After 15 minutes heating on the steam bath, the ferric hydroxide was removed by filtration and to the clear filtrate enough 10 per cent hydrochloric acid was added to obtain maximum precipitation of the amino compound. The precipitate was filtered off on a Buchner funnel after standing in the ice box overnight. It was freed from a very small amount of acid-insoluble material by redissolving in a small amount of water with a slight excess of dilute hydrochloric acid and reprecipitating from the filtrate by addition of the required amount of dilute sodium hydroxide. After standing in the ice box overnight, the substance was filtered off and dried *in vacuo* at 50° over calcium chloride. 70 to 80 per cent of the theoretical yield was obtained.

Para-aminomalonanilic acid ( $NH_2-C_6H_4-NH-CO-CH_2-COOH$ ): Crystallized from hot water using norit for decolorizing. White microscopic platelets. Melting point: 175–176°C., with gas formation.



Kjeldahl determination after drying at  $100^{\circ}$  *in vacuo* over sulfuric acid:  $C_9H_{10}O_3N_2$  calculated N 14.43 per cent, found 14.31 per cent.

Para-aminosuccinanic acid ( $NH_2-C_6H_4-NH-CO-CH_2-CH_2-COOH$ ): Crystallized from 15 parts of boiling water, using norit for decolorizing. Long, narrow, microscopic platelets. Melting point:  $183-184^{\circ}C.$ , with darkening.

Kjeldahl determination after drying at  $100^{\circ}$  *in vacuo* over sulfuric acid:  $C_{10}H_{12}O_3N_2$  calculated N 13.46 per cent, found 13.30 per cent.

Para-aminoglutaranilic acid ( $NH_2-C_6H_4-NH-CO-CH_2-CH_2-CH_2-COOH$ ): Crystallized from boiling water, using norit for decolorizing. Microscopic platelets. Melting point:  $186-187^{\circ}C.$ , with darkening.

Kjeldahl determination after drying at  $100^{\circ}$  *in vacuo* over sulfuric acid:  $C_{11}H_{14}O_3N_2$  calculated N 12.61 per cent, found 12.20 per cent.

Para-aminoadipanic acid ( $NH_2-C_6H_4-NH-CO-CH_2-CH_2-CH_2-CH_2-COOH$ ): Crystallized from 15 parts of boiling water, using norit for decolorizing. Long, narrow, microscopic platelets. Melting point:  $165-166^{\circ}C.$

Kjeldahl determination after drying at  $100^{\circ}$  *in vacuo* over sulfuric acid:  $C_{12}H_{16}O_3N_2$  calculated N 11.86 per cent, found 11.57 per cent.

Para-aminopimelic acid ( $NH_2-C_6H_4-NH-CO-CH_2-CH_2-CH_2-CH_2-CH_2-COOH$ ): Crystallized from boiling water, using norit for decolorizing. Long, narrow, microscopic platelets. Melting point:  $178-179^{\circ}C.$ , with darkening.

Kjeldahl determination after drying at  $100^{\circ}$  *in vacuo* over sulfuric acid:  $C_{13}H_{18}O_3N_2$  calculated N 11.20 per cent, found 11.06 per cent.

Para-aminosuberanic acid ( $NH_2-C_6H_4-NH-CO-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-COOH$ ): Crystallized from 100 parts of boiling water, using norit for decolorizing. Long, narrow, microscopic platelets. Melting point:  $162-163^{\circ}C.$

Kjeldahl determination after drying at  $100^{\circ}$  *in vacuo* over sulfuric acid:  $C_{14}H_{20}O_3N_2$  calculated N 10.61 per cent, found 10.38 per cent.

Para-aminooxanilic acid ( $NH_2-C_6H_4-NH-CO-COOH$ ): Prepared by condensation of para-phenylenediamine and oxalic acid following the methods described by G. Koller (10) and by W. A. Jacobs and M. Heidelberger (11). The product thus obtained was redissolved in about 30 parts of water with a slight excess of dilute sodium hydroxide. After making neutral to litmus, the solution was heated and decolorized with norit. The colorless filtrate was made faintly acid to Congo by addition of hydrochloric acid and the precipitated para-aminooxanilic acid was filtered and dried *in vacuo* at  $50^{\circ}$ .

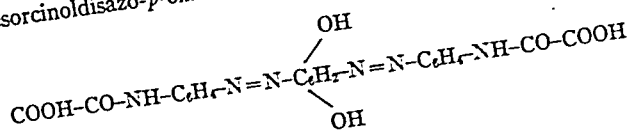
*Preparation of Azodyes.*—Resorcinoldisazo-*p*-anilic acids: 2 millimols of aminoanilic acid were dissolved in 50 cc. of water and 6 cc. of normal hydrochloric acid and diazotized at  $0-5^{\circ}C.$  by slow addition of 2 cc. of normal sodium nitrite. After the diazotization was complete (test with starch iodide paper), 1 millimol of resorcinol (110 mg.) dissolved in 40 cc.  $N/2$  sodium carbonate solution, cooled to  $0-5^{\circ}$ , was rapidly added with stirring, and the mixture was kept at  $0-5^{\circ}$  for  $\frac{1}{2}$  hour. Enough dilute hydrochloric acid was added to make the solution weakly acid to Congo and bring about complete precipitation of the dye, which was then separated from the liquid by centrifugalization. After washing twice

## K. LANDSTEINER AND J. VAN DER SCHEER

in the centrifuge tube with 50 cc. of water, the dye was redissolved in 40 cc. of water by addition of a slight excess of normal sodium hydroxide and was reprecipitated by the addition of hydrochloric acid. It was again washed three times with 50 cc. of water and dried at 75° *in vacuo* over calcium chloride. The yield was from 97 to 99 per cent of the theory.

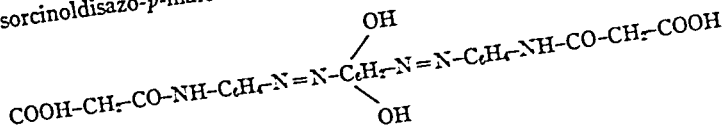
The following values for nitrogen found by analysis are calculated for ash-free substance. The content of ash in the dyes varied between 1 and 2 per cent with the exception of the resorcinoldisazo-*p*-oxanilic acid which contained 6.2 per cent ash.

Resorcinoldisazo-*p*-oxanilic acid:



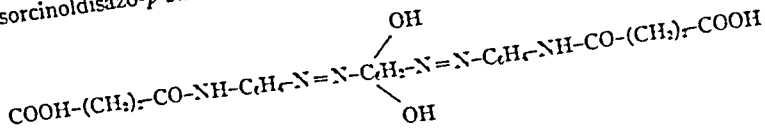
Calculated N 17.01 per cent, found 16.89 per cent.

Resorcinoldisazo-*p*-malonanilic acid:



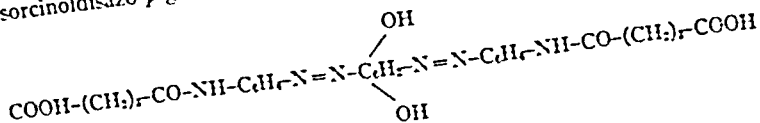
Calculated N 16.16 per cent, found 16.01 per cent.

Resorcinoldisazo-*p*-succinanilic acid:



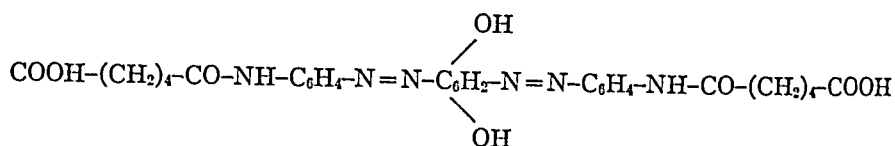
Calculated N 15.33 per cent, found 15.56 per cent.

Resorcinoldisazo-*p*-glutaranilic acid:



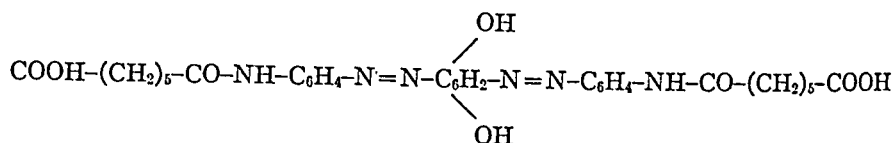
Calculated N 14.58 per cent, found 14.35 per cent.

Resorcinoldisazo-*p*-adipanic acid:



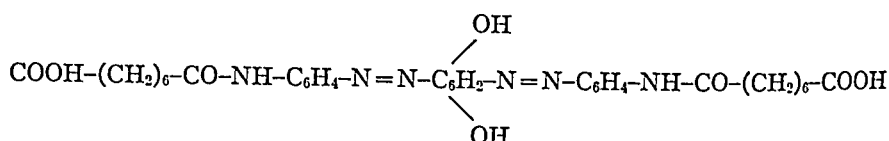
Calculated N 13.91 per cent, found 14.03 per cent.

Resorcinoldisazo-*p*-pimelanic acid:



Calculated N 13.29 per cent, found 13.10 per cent.

Resorcinoldisazo-*p*-suberanic acid:



Calculated N 12.73 per cent, found 12.81 per cent.

*Preparation of Azoproteins.*—1 millimol of aminoanilic acid was diazotized as described before at 0–5° and the diazo solution added to a cold solution containing 540 mg. of horse serum-globulin (prepared from diluted horse serum by half-saturation with ammonium sulfate and subsequent dialysis to remove the salt) and 13 cc. of normal sodium carbonate. The total volume of the mixture was approximately 65 cc. Coupling was allowed to proceed for  $\frac{1}{2}$  hour at 0–5° and the azoprotein was precipitated by addition of enough hydrochloric acid to make the solution weakly acid to Congo. The precipitate was separated from the liquid by centrifugalization and was washed several times with weakly acidulated saline. It was redissolved in saline by means of dilute sodium carbonate, making the solution neutral to litmus. As a preservative, 0.25 per cent phenol was added. The solutions used for the injections contained 5 mg. of azoprotein pro cc.

*Immunization.*—Four azoproteins were used for immunization; namely, those prepared from *p*-aminooxanilic acid, *p*-aminosuccinanilic acid, *p*-aminoadipanic acid, and *p*-aminosuberanic acid by coupling to horse serum-globulin as described above. For the immunization with each antigen four rabbits were used, each receiving daily intravenous injections of 2 cc. of the antigen solution for 6

days. One or two more courses of injections were given at intervals of 1 week,<sup>2</sup> and the sera were tested 7 days after the last injection. Two to three active sera were obtained in each series. For convenience the sera will be referred to as oxanilic, succinanilic, adipanilic, and suberanilic immune sera.

#### EXPERIMENTAL

*Precipitin Reactions with Azodyes.*—The immune sera obtained in the manner described, with the exception of the oxanilic immune sera, gave distinct precipitin reactions when added to solutions of the sodium salts of the corresponding resorcinol-azodyes (Table I).<sup>3</sup>

Results practically identical with those recorded in the table were obtained with the other sera at our disposal.

The reactions with the succinanilic sera were very specific whereas the action of the immune sera for the antigens with longer side chains extended to the compounds next in the series. The same sort of specificity appeared in tests with azoproteins. These observations will be described in a subsequent communication.

The strongest reactions occurred with the suberanilic immune sera and the homologous dye. Upon the addition of the antiserum, a distinct turbidity appeared almost immediately and after  $\frac{1}{2}$  hour small flakes, and later larger ones were formed, the phenomenon resembling in all respects a common precipitin reaction. The highest dilutions in which a reaction was still noticeable corresponded to a content of 0.001 mg. per 1 cc.; consequently, the sensitivity of the tests is of the same order as precipitin reactions with proteins or bacterial carbohydrates. In the tests with the succinanilic and adipanilic immune sera the reactions were similar, but the precipitation developed more slowly and was less intense. Whilst in the case of the adipanilic sera this difference is probably due to the fact that the immune sera happened to be less active, as evidenced by the reactions with azoproteins, this explanation does not apply to the succinanilic sera which were almost as active as those for the suberanilic compound. Probably the reason for the weaker reactions is a lower precipitability of the dyes due to the shorter side chain. This assumption is borne out by the observation that other azodyes examined were far less precipitable

<sup>2</sup> Cf. Reference 12.

<sup>3</sup> Reactions were also obtained by complement fixation.

TABLE I

1/50 millimol of the dye (for instance 13.2 mg. of the resorcinoldisazo-*p*-suberanilic acid) was dissolved in 3 cc. of N/50 sodium hydroxide and 1 cc. N/50 hydrochloric acid and 1 cc. distilled water was added. The solution was centrifuged to remove traces of insoluble material. In the case of resorcinoldisazo-*p*-oxanilic acid, 1 cc. water was added in place of hydrochloric acid since it was found that the greater alkalinity was necessary to prevent flocculation upon subsequent dilution with saline.

For the precipitin tests given in Table I, 1 to 3 capillary drops of immune serum were added to 0.2 cc. of the dye solutions (prepared as described above), diluted 1:20, 1:100, and 1:500, with saline. Readings were taken after 1 and 2 hours at room temperature and after standing overnight in the ice box. The intensity of the reactions is indicated as follows: 0, f. tr. (faint trace), tr. (trace), tr. (strong trace),  $\pm$ ,  $\pm$ ,  $\pm$ ,  $\pm$ ,  $\pm$ , etc.

Control tests with normal rabbit sera and some other immune sera such as precipitins for human or pig serum gave no reactions.

Immune sera for azoproteins made from	Readings taken after	Dyes made from											
		<i>p</i> -Aminooxanilic acid, dilution			<i>p</i> -Aminomalon- anilic acid, dilution			<i>p</i> -Aminosuccin- anilic acid, dilution			<i>p</i> -Aminoglutar- anilic acid, dilution		
		1:20	1:100	1:500	1:20	1:100	1:500	1:20	1:100	1:500	1:20	1:100	1:500
<i>p</i> -Aminosuccin- anilic acid, 2 drops	1 hr.	0	0	0	0	0	0	0	0	0	0	0	0
	2 hrs.	0	0	0	0	0	0	0	0	0	0	0	0
	Night in ice box	0	0	0	0	0	0	$\pm$	$\pm$	$\pm$	0	0	0
<i>p</i> -Aminoadip- anilic acid, 3 drops	1 hr.	0	0	0	0	0	0	0	0	0	0	0	0
	2 hrs.	0	0	0	0	0	0	0	0	0	0	0	0
	Night in ice box	0	0	0	0	0	0	0	0	0	$\pm$	f. tr.	0
<i>p</i> -Aminosuber- anilic acid, 1 drop	1 hr.	0	0	0	0	0	0	0	0	0	$\pm$	tr.	0
	2 hrs.	0	0	0	0	0	0	0	0	0	$\pm$	tr.	0
	Night in ice box	0	0	0	0	0	0	0	0	0	$\pm$	$\pm$	f. tr.

The dilutions are in terms of a solution containing 1/50 millimol of the dye in 5 cc.

than the resorcinoldisazo-suberanilic acid.<sup>4</sup> Azodyes made by coupling 2 mols diazotized dextro-para-aminotartranilic acid or para-saranilic acid with 1 mol resorcinol, in dilutions of 1:100 and 1:500 of 1/50 millimol of the dye in 5 cc., gave only very weak reactions with the homologous immune sera (1, 8) upon standing for 2 hours at room temperature and overnight in the ice box. On subsequent

TABLE II

0.2 cc. of a solution of the sodium salts of the resorcinol dyes in saline (containing 1/2500 millimol in 10 cc.) were mixed with 0.05 cc. of a neutral solution of the sodium salts of the nitroanilic acids, containing 1/32 millimol in 10 cc. for the tests with the suberanilic immune serum and 1/64 millimol in 10 cc. for the tests with the succinanilic and adipanilic immune sera. 1 to 2 drops immune serum were added. The control tube contained the dye solution, immune serum and 0.05 cc. saline. The readings were taken after 1 and 3 hours at room temperature and after standing overnight in the ice box.

Immune sera for azoproteins made from	Readings taken after	Substances				
		<i>p</i> -Nitro- oxanilic acid	<i>p</i> -Nitrosuc- cinanilic acid	<i>p</i> -Nitro- adipanilic acid	<i>p</i> -Nitro- suberanilic acid	Control
<i>p</i> -Aminosuccinanilic acid, 1 drop	1 hr.	+	0	±	±	+
	3 hrs.	+	0	+	+	+
	Night in ice box	++±	0	++±	++±	++±
<i>p</i> -Aminoadipanilic acid, 2 drops	1 hr.	±	±	0	tr.	±
	3 hrs.	+	±	0	tr.	+
	Night in ice box	±±	+	0	±	±±
<i>p</i> -Aminosuberanilic acid, 1 drop	1 hr.	±±	±±	+	0	++
	3 hrs.	++	±±	+	0	++±
	Night in ice box	++++±	++±	++	0	++++±

spinning the reactions became much more evident, the precipitate appearing as large, thin flakes upon shaking up the sediment.

The tests presented in the table were made with freshly prepared solutions of the sodium salts of the dyes. When the solutions (con-

<sup>4</sup> In this connection the results of Hartley (13) on the precipitation of defatted proteins are worth mentioning.

taining 1/50 millimol of the dye in 5 cc.) were kept in the ice box for several days up to a week, the precipitability with immune serum increased gradually, probably owing to a lowered dispersion of the dissolved substance. These solutions were made as described above and contained only a very small quantity of inorganic salt. For the tests they were diluted with saline in the usual way.

From the results reported one would anticipate the possibility of eliciting anaphylactic shock in animals sensitized with azoprotein, by administration of those dyes which are distinctly precipitated by immune sera. Such was indeed the case; it is intended to describe these experiments later in detail.

*Inhibition of the Precipitin Reaction.*—The similarity between the precipitin reaction with dyes and those with azoproteins extends to the phenomenon of inhibition by low molecular substances with corresponding groupings. Thus neutral solutions of the sodium salts of nitroanilic and aminoanilic acids inhibited the precipitin reactions with the dyes specifically. Such results are presented in Table II. The solutions of nitro- or aminoanilic acids were not precipitated by the immune sera.

#### COMMENT AND SUMMARY

Experiments are described demonstrating the precipitation of azo-dyes by immune sera prepared by the injection of azoproteins containing the same azo component. These precipitin reactions prove conclusively the view already advanced on the basis of inhibition reactions that antibodies combine specifically with substances of small molecular weight. Although in this respect both phenomena have the same significance, the precipitin reactions with dyes are simpler in that the aid of a protein antigen is eliminated.

That specific serological precipitin reactions can take place with substances other than proteins has been amply demonstrated by studies on bacterial antigens (14) (polysaccharides (15)). The present findings show that for the precipitation with immune sera not even a high molecular weight of the reactive substance is required. Factors determining the tendency to separate out from the liquid upon combination with antibody seem to be the colloidal state of the solution and the chemical composition of the substance. With regard to the

influence of chemical composition, a striking example is provided by the suberic acid dye which gives particularly strong precipitin reactions, most probably on account of its long aliphatic side chains.

The results reported may be of use for studies on the mechanism of serological precipitation.

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# THE INDUCED DEVELOPMENT OF NON-ACID-FAST FORMS OF BACILLUS TUBERCULOSIS AND OTHER MYCOBACTERIA\*

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In a previous communication (1), a description was given of an organism which was obtained from a stock culture of human tubercle bacilli, Strain H-37. This organism had the morphological characteristics of the parent strain but its growth was more rapid, and it was chromogenic, having acquired a deep yellow-orange color. On media suitable to acid-fast growth, colonies of this organism were made up of non-acid-fast rods with a scattering of acid-fast granules and rods. On more simple media, the acid-fast elements were almost entirely absent. This organism caused tuberculosis in three of five guinea pigs. It was assumed to be a non-acid-fast type of *B. tuberculosis* and was named the chromogenic H-37 strain.

The work represented in the present communication was done in order to determine whether there existed in association with the chromogenic H-37 strain an agent causing the loss of acid-fastness and, if this agent was transferable, to use it in degrading acid-fast organisms into non-acid-fast forms.

Extracts of the chromogenic H-37 organism were made and passed through a Berkefeld filter and added to the culture media on which six strains, representing four types of mycobacteria, were grown. From five of the six strains, growths which were apparently wholly non-acid-fast were obtained; the sixth strain, an organism recently isolated from human renal tuberculosis, developed non-acid-fast

\* This work was begun in the laboratories of The Rockefeller Institute for Medical Research, New York.

ments which were not readily separated from the acid-fast ones. Some of the non-acid-fast growths which appeared following treatment of the parent organism with the extract from the chromogenic 37 organisms have by cultural methods been brought to yield acid-fast organisms again.

The history of non-acid-fast tubercle bacilli dates back almost to the original discovery of *B. tuberculosis* by Koch. In 1883, Malassez and Vignal (2) discovered non-acid-fast coccoid forms in zooglyphic masses in skin lesions of a tuberculous patient and recovered acid-fast rods from them by animal passage. Ferran (3) also found that a reduction in the amount of glycerine in the media caused a reduction in the acid-fast property of the organisms and a lowering of their virulence. Auclair (4) repeated Ferran's work except that he used no glycerine in the media and was able to change a culture of tubercle bacilli to non-acid-fast coccoid and rod forms which were avirulent. He could not make this culture regain its virulence.

Further studies of tubercle bacilli during the early years (5-23), many of them dealing with the effects of variations in culture media, served to bring out the marked pleomorphism and the variability in staining reaction of this group of organisms. Of these studies it is interesting to note that Nocard and Roux were the first to find branching forms and growth by budding; that Schürmayer called attention to branching forms which were found originating in cultures of avian tubercle bacilli; d'Arrigo described extreme variation in the types of coccobacteria occurring in scrofulous lymph nodes; Marmorek stained the tiny colonies that first spread out from the edge of cultures of tubercle bacilli on liquid media before any of the characteristic wrinkling of the surface had taken place and found many non-acid-fast forms; and Spengler observed in sputum acid-fast colonies which he called "splitters." They were smaller than tubercle bacilli.

Koch had seen in tubercle bacilli both granules and certain refractive bodies which for a time he considered to be spores, but only a little attention had been given to either until the work of Much (24) on the granules. He found that in material such as that of cold abscesses and certain samples of sputum, in which it is impossible to demonstrate acid-fast organisms and yet which could be proved tuberculous by inoculation into animals, there were Gram-positive granules, since he called Much granules. He also followed tuberculous sputum day by day and found that when acid-fast organisms disappeared, these granules could be demonstrated. He believed that typical acid-fast bacilli could be reproduced from them. Engel (25), on the other hand, believed that these granules should be considered as forms of degeneration. Much's work was first discussed largely from the standpoint of the relations of the granules to diagnosis, but at the present time the work is of greater interest in relation to certain general biological problems such as the nature of the life cycle of bacteria and the question of filterability. In the mycobacteria the determination of the occurrence of a non-acid-fast phase is a funda-

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mental point in the study of whether the life cycle of these organisms is simple or complex.

The extensive literature of the past 20 years on the growth of tubercle bacilli may well be considered in connection with how much light it throws on the life cycle of these organisms. It has been analyzed recently by Löhnis (26), Sweany (27), and Kahn (28, 29). The most extensive bibliography is the recent list of 462 articles by Puntoni (30).

In 1912, Krylow (31) extended the earlier work of Marmorek and gave evidence to show that tubercle bacilli were at first non-acid-fast and Gram-negative; next developed the Gram-positive material which was always in the form of granules, and finally the acid-fastness which he thought appeared diffusely in the bacilli. The next year Wherry (32) cultivated non-acid-fast coccoid, rod, and spore-like forms from a saprophytic type of tubercle bacilli and from them regained acid-fast forms. Meirowsky (33) gave extensive illustrations of the vitally stainable granules of human and bovine tubercle bacilli and lepra organisms with studies of the growth of tubercle bacilli by budding.

More recently the studies have been more definitely along the line of a complex life cycle for the mycobacteria (34-47). In 1931, Maher summarized several years of work which had enabled him to cultivate non-acid-fast forms from old cultures of acid-fast growths. Bezançon and Philibert took the sections with the Ziehl-Neelsen technique and in these sections found dense networks of non-acid-fast material from which acid-fast rods and granules were growing. Ravetlat and Pla y Armengol summed up their work as indicating three phases in the life cycle of tubercle bacilli; first, a "bacterium of attack" made up of non-acid-fast forms, which were cocci, diplococci, tetrads, chains of cocci, and zooglyc masses; second, intermediate forms to be obtained from caseous material, including non-acid-fast bacilli, the Much granules, and certain intracellular forms; and third, the Koch bacillus, which they consider to be a "resisting form."

Vaudremer showed that tubercle bacilli, both human and bovine, when grown on media poor in nutritive value and without glycerine, became non-acid-fast and very pleomorphic and lost their power to produce tuberculin. In this state they ranged from a mycelium-like form to zooglyc masses. On suitable media these organisms became acid-fast again. Sweany made extensive studies of the granules and found that they could sprout and give rise to acid-fast organisms. When single-celled the granules could reproduce cultures of typical acid-fast organisms. In small doses the granules gave atypical chronic lesions and in more massive doses they gave typical tubercles. He considered them as variants in the life cycle of tubercle bacilli.

Kahn has presented the most convincing evidence of a complex life cycle of tubercle bacilli. He isolated single organisms from acid-fast cultures of tubercle bacilli, Strain H-37, planted them in microdroplets, and found that these bacilli divided into cocci which then became colonies of exceedingly small organisms which were non-acid-fast and which ultimately grew into acid-fast bacilli like the

original organism. Oerskov has again raised the question of degenerative forms in the cycle as described by Kahn on the basis that similar forms can be seen in autoclaved cultures. He did not recognize that Kahn had produced tuberculosis in animals from the single cell cultures. From these studies it is clear that a complex life cycle, involving a non-acid-fast phase, has been reported repeatedly, but the difficulties involved in ruling out contaminants in a convincing manner have prevented any general acceptance of the existence of such a cycle.

### *Methods and Technique*

The chromogenic H-37 strain was grown on plates of Petroff's egg media. At the height of the growth, or when the pigment was fully developed, the organisms were removed from the plates. Each plate yielded about 1.5 gm., which was placed in a sterile mortar. After the addition of 15 cc. of sterile saline or sterile distilled water, the material was ground for 15 minutes and then filtered through N Berkefeld candles. The filtered extracts were then placed in sterile containers and kept in the ice box. They were tested for sterility when used.

The six strains of mycobacteria used in these experiments included three human strains—Saranac H-37, T. S., and 90—, a bovine Strain B-1, a smegma Strain 74, and a Saranac strain of *B. phlei*. Three of these—H-37, T. S., and B-1—were proved to be virulent for animals. The smegma and *phlei* strains were both saprophytic, and 90 was isolated from human renal tuberculosis.

Stock cultures of all of these were grown on Petroff's egg media, and transplants were made every 4 to 6 weeks. The strain to be used for an experiment was usually of a 10 day growth. The procedure, except in two or three instances, was to add 1 to 10 cc. of filtered extract to a fresh transplant of this growth. The excess fluid was allowed to dry (in the incubator) before the culture was sealed or banded. In several experiments, a second and a third addition of the extract were made to the culture before microscopic or macroscopic changes were noted. Each time the extract was added to the culture, a control plate or tube of medium was prepared by the addition of the extract to it.

The treated cultures were transplanted in 5 days, 2 weeks, 4 weeks, and 6 weeks, with a few exceptions. The number of non-acid-fast elements which had developed prior to each transplant determined further treatment with the extract. Transplants from the originally treated cultures were made on Petroff's egg media, or on the usual laboratory media such as plain or blood agar.

After the development of many non-acid-fast forms, the organisms were grown on agar until cultures were obtained which appeared in smears to be entirely non-acid-fast. When this was achieved, they were inoculated onto media suitable for the cultivation of acid-fast growth, for example, Petroff's egg media.

### *Action of the Extract*

When the extract was added to Petroff's egg media, the action on many cultures of the acid-fast strains mentioned was shown by typical

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macroscopic and microscopic changes. There was acceleration of growth of the fresh transplant within 5 days. In smears at this time many non-acid-fast organisms were seen. At 2 weeks the culture originally treated still showed more rapid growth than usual, but fewer non-acid-fast elements than at 5 days.

At about the 4th to 6th week the growth of treated cultures suddenly became fatty and moist in appearance. In some of these cultures the colonies appeared shrunken. At this time, the acid-fast elements were similar to those of old cultures: many were in the form of beaded rods, many were granules, and others only weakly acid-fast; there were also clumps of material without bacterial form. The non-acid-fast forms were often equal in numbers to the acid-fast ones and were either coccoid or bacillary. One culture, B-1 92, showed branching forms with small acid-fast granules lying along and at the ends of the organisms. From the findings it is clear that the growth of these organisms on Petroff's egg media after the use of the extract has been extremely pleomorphic. All of these strains of mycobacteria, H-37, T. S., 90, B-1, 74, and the *B. phlei* strain, have shown these peculiar changes. In the case of the strain of *B. phlei*, which normally grows rapidly on agar, the culture, treated with extract and transplanted to agar, showed acceleration of growth, loss of pigment, and many non-acid-fast forms; yet repeated transfers were necessary to eliminate the acid-fast forms.

After these peculiar macroscopic and microscopic changes had occurred, or usually after the 6th week, non-acid-fast cultures generally were grown with ease from transplants to agar.

In order to test the effect of the extract on existing acid-fast organisms, the following experiment was done. H-37 tubercle organisms were placed in 2 cc. of the extract and incubated for 96 hours. During this time, smears and cultures were made after 1 hour, 24 hours, 72 hours, and 96 hours. No increase in the non-acid-fast forms was found at these intervals, but from each transplant non-acid-fast forms were grown.

During the time that these macroscopic and microscopic changes occurred in treated cultures, the control plates remained sterile. In addition to the control plates, the sterility of the extracts was indicated by further tests on plain agar, plain broth, infusion broth, and

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TABLE I  
*Protocol of an Experiment—(Series 72)*

Culture No.	Date of trans-plant	Media	Type of growth	Acid-fast elements	Non-acid-fast elements	Remarks
51-II	1931-32	Petroff's egg	Normal	++++	0	Virulent for animals
1 (from Stock H-37, 51-II)	June 5	Long's + 10 cc. of extract (chromogenic H-37 <i>B. tuberculosis</i> )	Slight growth of pellicle in 3 wks. Plant sank to bottom. Gray-white, mucoid growth in 4 wks.	+	+++	Almost entirely non-acid-fast cocci and rods
2 (from No. 1)	July 2	Plain agar	Glistening, gray-white, pinpoint colonies in 24 hrs.	0	++++	Entirely non-acid-fast cocci and rods; rods with small, round, non-acid-fast inclusion bodies
3 (from No. 2)	July 4	Petroff's egg	Moist, fatty, yellow-white growth in 6 days	0	++++	Same as above (No. 2). Placed in ice box July 10 for 6 wks. Placed in incubator Aug. 24, still non-acid-fast. Acid-fast granules appeared by Sept. 8
4 (from No. 3)	Sept. 8	Plain agar	Glistening, gray-white, pinpoint colonies	+	+++	Biweekly transplants carried on agar through 5 mos. became and remained wholly non-acid-fast, showing cocci, rods, and diphtheroids
4a (from No. 3)	Sept. 8	Petroff's egg	Moist, fatty colonies, otherwise similar to mycobacterial growth	++	++	Few acid-fast rods. Many non-acid-fast rods and granules, many of these rods with acid-fast inclusion bodies

5 (from No. 4a)	Oct. 6	Petroff's egg	Similar to No. 4a, but drier	++	++	Equal distribution of acid-fast rods and granules and of non-acid-fast rods and granules
5a (from No. 4)	Oct. 6	Potato glycerine broth	Pellicle formation	+	+++	Mostly acid-fast rods. Few non-acid-fast rods and granules
6 (from No. 5a)	Nov. 20	Petroff's egg	Rapid, moist, fatty growth. Became drier, heaped up, and worm-like in appearance	++	++	Equal distribution of acid-fast rods and granules, and of non-acid-fast rods and granules
7 (from No. 6)	Jan. 14	Petroff's egg	Rapid, flat, yellow, mucoid growth in 6 days. In 2 wks., dry, waxy, heaped up appearance, typical of Hf-37	+	+++	At 6 days mostly non-acid-fast rods and cocci. At 2 wks. almost entirely acid-fast
Control 1	Planted June 5	Petroff's egg + 2 cc. of extract	—			No growth. Discarded Aug. 24
Control 2	Planted June 5	Petroff's egg + 2 cc. of extract	—			No growth. Discarded Aug. 24

Kendall's K medium. The K medium culture was kept sealed for 3 weeks and during that time remained sterile; it was transplanted to infusion broth and during 3 additional weeks remained sterile.

In Table I an experiment and its results are summarized, the changes being those which occurred in successive subcultures of the H-37 strain of human tubercle bacilli after the initial culture had been treated with the extract. The first culture of the experiment was planted on Long's medium and did not show the initial acceleration of growth of other treated cultures. Otherwise, the changes which occurred were similar to those in other experiments. It will be seen in the table that 4 weeks after the bacteria had been placed in contact with the extract, a non-acid-fast organism, which grew rapidly on agar, was obtained. The sequence demonstrated on the table includes the return to a morphology similar to the original strain. It also includes a notation of the continued growth on agar of the non-acid-fast forms.

From five of the six acid-fast strains employed, there were produced non-acid-fast strains. The sixth, No. 90, showed the macroscopic and microscopic changes noted above, but thus far we have been unable to eliminate all of the acid-fast forms. Four of these strains have been changed from two to six times. H-37 has yielded six non-acid-fast growths; B-1, three; T. S., two; and 74 has yielded three non-acid-fast growths.

Recently, we have also produced a non-acid-fast strain of H-37 and one of T. S., by treating cultures of these organisms with autoclaved extracts of the chromogenic H-37 strain. In all, seventeen wholly non-acid-fast growths have been obtained. All of these grew rapidly on agar and at first all but one of these growths were made up of coccoid and rod forms which were both acid-fast and non-acid-fast.

#### *Types of Non-Acid-Fast Growth*

The first isolated culture of non-acid-fast forms from a treated culture was usually a small, fine, glistening, gray-white growth. This growth, usually a transplant from Petroff's egg media to plain agar, appeared on the agar in 24 hours but was not well developed before the end of 48 hours. Microscopically, the growth usually was made up of cocci, a few of which were very large, and of rods. The cocci were often in diplococcus or tetracoccus forms, although some short

chains were found. At first among the non-acid-fast forms there were found acid-fast granules and rods, as well as transition forms such as non-acid-fast rods containing acid-fast bodies, chains of acid-fast granules held together by non-acid-fast filaments, and faintly acid-fast cocci. Thus, the growth was increasingly pleomorphic.

When grown on glucose broth or infusion broth, there was a tendency to diphtheroid formation, but this did not always occur. On infusion broth and blood agar, there was also occasionally a shift back to acid-fastness even if the growth had been transplanted on agar several times.

Other types of non-acid-fast growth were observed; for instance, from a treated culture of B-1 (mentioned above), a branching non-acid-fast form was isolated. Four cultures, which were isolated as non-acid-fast cocci and rods after different periods of time on agar, changed to a more rapid, luxuriant growth which was mucoid. These growths were made up of rods containing spore-like bodies and many free spore-like bodies. When free, they were somewhat acid-fast. These bodies appeared either in the center or at the ends of the rods. One culture of chromogenic H-37, which was transferred weekly on agar for a period of 2 months, also developed a mucoid appearance. This growth showed spore-like bodies both in rods and free. Transplants of this organism were made on agar, blood agar, and Petroff's egg media. On the agar, the rods containing spore-like bodies continued to develop, the blood agar culture reverted to cocci, diphtheroids, and small rods, all non-acid-fast (the normal appearance of chromogenic H-37 on blood agar); on the Petroff's egg media, the culture returned to small non-acid-fast rods and a few acid-fast rods, and to its original appearance with deep yellow-orange pigment.

#### *Return to Acid-Fastness*

The return of the non-acid-fast organisms to an acid-fast state was a slow process. After the non-acid-fast forms had been transferred on agar for from four to eight times, they were transplanted to Petroff's egg media or other media suitable for the development of acid-fast growth. Of the seventeen non-acid-fast growths only four have returned to acid-fastness thus far. These were two cultures of H-37 (72 and 822), smegma 74, and *B. phlei*, and they have returned not

only to acid-fastness but the organisms are similar to the original strain, except that they still contain a number of non-acid-fast forms. In the case of *B. phlei*, the non-acid-fast forms in the culture which returned to acid-fastness were no greater in number than those usually found. The reappearance of macroscopic morphological characteristics included the chromogenicity of *B. phlei*, and the rapid, cream-colored growth of smegma.

This return to acid-fastness has been through the development of acid-fast granules among the non-acid-fast cocci, an agglomeration of these granules into chains which resemble somewhat the beaded acid-fast rods of old cultures, the appearance of acid-fast globules within non-acid-fast rods, and finally the development of acid-fast rods. In the colonies in which acid-fast rods were developing, transition forms were always present in varying numbers. Transplants which were made when these forms predominated caused a temporary reversion to non-acid-fast forms, showing the instability of the culture.

#### DISCUSSION

The question of whether there exists a non-acid-fast phase of the mycobacteria is fraught with many difficulties. In this study, one source of error has been eliminated in the use of the extract from the chromogenic H-37, namely, the possibility that living organisms have been added. In the first place, all of the preparations of extract had been passed through a Berkefeld filter and the filtered extracts were all negative for viable organisms, as shown by consistently negative cultures on several standard media. Moreover, the extract was just as effective after it had been autoclaved, thus ruling out the possibility that it was a carrier of living contaminants.

Concerning the possibility of contaminations of the original cultures, or of those developing during the course of the transplantation, with some non-acid-fast saprophyte which might grow better on the simpler media but survive on media adapted to acid-fast growth, it is more difficult to bring forward convincing evidence. The facts in favor of the view that the cultures we have been working with are those of uncontaminated mycobacteria are as follows:

First, the original cultures of acid-fast organisms all showed the colony formation typical for mycobacteria and the strains all appeared

to be growing in pure culture. No more non-acid-fast elements were found in smears of the original cultures than are normally found in smears of acid-fast cultures, that is to say, it required diligent search to discover even one or two non-acid-fast forms. If, however, the tubercle bacillus, at some time in its life cycle, has a non-acid-fast phase, as Kahn's observations indicate, so that not every single tubercle bacillus results from the direct fission of an acid-fast organism, then some non-acid-fast forms may be present always in any pure culture of tubercle bacilli. Second, experiments carried out in Cleveland gave the same results as in New York, so that if the non acid-fast forms were contaminants it would have to be postulated that stock cultures in the two places were carrying the same contaminants. Third, all of the organisms showed the same type of changes after the use of the extract, a change in rate of growth, in type of colony formation, and in the increased proportion of the non-acid-fast forms which were extremely pleomorphic. If contaminating saprophytes were involved which overgrew the mycobacteria on plain agar, then it would be necessary to assume that the same contamination accompanied all the strains studied in two laboratories. Fourth, frequent attempts were made by the method of dilution cultures to separate the non-acid-fast forms from the acid-fast when both were present, but we were never able, by this method, to obtain colonies which were either wholly acid-fast or wholly non-acid-fast. This is best explained by the study of the colonies themselves, for they were usually made up not only of a mixture of the two forms, but some of the non-acid-fast rods contained acid-fast particles. The development from a completely non-acid-fast culture of these transition forms, partly acid-fast and partly non-acid-fast, and the subsequent return of the culture to acid-fastness are the best evidence that the wholly non-acid-fast forms in our cultures were not contaminations.

Final evidence is to be obtained by applying the technique of the single cell culture to this problem. By this method we have already obtained a growth from a single organism isolated from an acid-fast culture. This bacillus was planted on media to which the extract had been added. The growth resulting was partly acid-fast and partly non-acid-fast. The organism grew both on agar and on Petroff's egg media and from the latter acid-fast rods have been obtained. In

future experiments not only must a single cell be taken many times from the original acid-fast strain but also from the non-acid-fast subcultures until adequate data have been obtained on the limits of variation of the mycobacteria. Extensive studies also must be made on the pathogenicity of these different forms.

The evidence up to the present time, namely the similarity of morphology of the non-acid-fast growths, the regularity with which they were obtained, the transition forms in the cultures, and the fact that four of the non-acid-fast growths when transferred to appropriate media have returned to acid-fast forms showing the original type of growth, makes it reasonable to assume that the non-acid-fast organisms were derived from acid-fast ones and that the phenomenon occurs regularly.

#### SUMMARY

Six strains of mycobacteria,—three human strains, Saranac H-37, T. S., and No. 90, a bovine strain, B-1, a smegma strain, No. 74, and a Saranac strain of *B. phlei*,—have been made to grow as non-acid-fast organisms by the addition to the culture media of a filtered extract of the chromogenic H-37 strain of *B. tuberculosis*. The action of the extract produced acceleration of growth of the treated culture, followed by macroscopic and microscopic changes, and differentiation into non-acid-fast forms. The bacterial forms grown from these treated cultures were pleomorphic, usually consisting of cocci and small rods; but branching forms and spore-like bodies also developed.

The sterility of the extract causing the changes was demonstrated by frequent control inoculations on various media, including Kendall's K medium; and autoclaved extracts had the same effects as non-autoclaved.

After transfer to media suitable for acid growths four of the strains reverted not only to acid-fastness but to their original cultural characteristics, providing evidence that the non-acid-fast forms were specific for the strain.

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red marrow spread becomes even more limited and we find less and less red marrow in the long bones and even some fat cells replacing red marrow in the cancellous bone of the ribs and vertebrae. As the red marrow spreads or shrinks the fat cells act as the cushion to take up or give space. With red marrow expansion the fat cells retire and as the red marrow shrinks the fat cells take its place in the cancellous bone lacunae.

In general we may say that conditions in the spleen and red marrow run along parallel as the normal dog grows up and with old age both marrow and spleen show atrophy. If the red marrow were a nicely isolated organ like the spleen its study would certainly become much less time consuming.

In long continued severe anemia in dogs one would expect a wider red marrow spread and such indeed will be observed in many cases described below. In some anemic dogs the red marrow fills practically all the cancellous bone in ribs, vertebrae and long bones and this is the reaction which would be expected because of the long continued maximal stimulus for red cell production (Figs. 1 and 2). But other dogs will show very much less red marrow spread, scarcely more than observed in normal non-anemic adults and yet such dogs will produce as much new hemoglobin as is produced by the dogs with grossly hyperplastic red marrow having a maximal red marrow spread (compare Dogs 24-2 and 25-24, Table 1).

Every degree of marrow hyperplasia is to be observed in this series of dogs from the 100 per cent of Dog 25-24 (Figs. 1 and 2) down to the minimal spread of Dog 18-114 (Figs. 3, 4 and 5). The details are all the same but the mass of the red marrow varies.

Evidently the red marrow varies in extent in normal non-anemic dogs. Given the stimulus of a severe anemia due to bleeding the red marrow may spread to include all the cancellous bone spaces in the skeleton or to include only a part of this large area. The maximal hemoglobin production of this hyperplastic marrow may be much the same in the two instances. It would seem that some other factor were concerned with the limitation of hemoglobin output and red marrow spread.

Evidence is accumulating to indicate that the liver may be this limiting factor—that the liver may be responsible for fabrication of some essential precursors of hemoglobin and that the capacity of the

liver may set the top limit for maximal red cell and hemoglobin production. This evidence comes in part from Eck fistula dogs whose hemoglobin and pigment production is usually subnormal (9). These Eck fistula dogs with progressive repeated injury of the liver may finally reach the stage where they can produce only sufficient red cells and hemoglobin for simple maintenance with complete obliteration of the usual surplus of hemoglobin production on a liver or kidney diet (experiments unpublished). The only abnormality is the injured liver so that this experiment would indicate that the liver is essential for normal hemoglobin and red cell production in the dog.

*Reserve storage of hemoglobin* is a subject of considerable interest. There is no doubt that there is a good deal of reserve hemoglobin stored in the normal dog which will be contributed to the circulation as experimental anemia due to bleeding is produced. Is this reserve stored as mature or immature red cells in the red marrow or spleen? Or is it stored as parent substances capable of being built up into the mature red cells as the emergency develops?

There is ample evidence that a standard anemic dog placed on a favorable diet (liver or kidney) will produce large amounts of new hemoglobin and red cells (6, 7). This production results from a 2 week feeding period and may amount to 100 gm. new hemoglobin over and above control bread diet periods. This new hemoglobin will appear in the circulation in the 1st week in small amounts and is removed by bleeding. Much more will appear in the 2nd week and is removed by bleeding. There is always a large carry over into the 2 weeks after period which may amount to half the total hemoglobin produced due to the liver or kidney diet. Therefore at the end of the 2 weeks liver feeding the dog may have in reserve somewhere in his body the materials from which he can produce 40 to 50 gm. hemoglobin during the after period. Where is this reserve stored? Is it stored as red cells or as intermediates in the marrow or parenchymatous organs?

If the reserve hemoglobin was stored in the form of mature red cells it would amount to 100 to 125 cc. of packed red cells. To store this amount of red cells in the dog's marrow is obviously a physical impossibility. Moreover there is no histological evidence of any marked increase of mature red cells in dog's marrow when this reserve storage is known to exist—see Dog 25-24.

Feeding experiments would indicate that some of these hemoglobin precursors under such conditions of maximal reserve storage (Dog 25-24) are stored in the liver and kidney rather than in the spleen. It must be admitted that much of this hemoglobin reserve is stored in some intermediate form which at the present time we cannot identify.

### *Methods*

All material studied and reported below came from the anemia colony in this laboratory. The dogs are bred in this laboratory and the dominant strain is that of a short-haired white bull dog. The stock is not pedigreed and there is some coach dog and terrier blood in the colony. These dogs unless otherwise noted are active and perfectly normal in all respects and live out a normal life cycle. The only abnormality is a blood hemoglobin level of 40 to 50 per cent instead of the normal for this colony of 140 to 150 per cent hemoglobin. The anemia level is maintained by appropriate bleedings each week and the hemoglobin removed is estimated and recorded in the tables as grams hemoglobin bled. This continued maximal stimulus to the formation of new hemoglobin and red cells leads to standard output figures for hemoglobin on various standard diets. Many reports of these diet studies and the resultant hemoglobin production have been published by Robscheit-Robbins and Whipple (10, 7).

The standard bread used in these experiments is made in the laboratory from wheat flour, potato starch, bran, sugar, canned salmon, cod liver oil, canned tomatoes, yeast and a salt mixture. This bread is a complete diet capable of maintaining dogs in health for indefinite periods; its preparation has been described (10).

The periods of anemia in the reported series vary from 1 year and 4 months to 7 years and 4 months. In some of the early experiments the bleeding was discontinued in the summer (July and August) but the anemia level maintained continuously during the rest of each year. The longest period of uninterrupted anemia was 5 years.

As dogs died or were killed by accident or by anesthesia, a routine autopsy was done. All long bones were split and notes made as to the gross appearance of the red and fat marrow. Sections from all organs, marrow of long bones, ribs and vertebrae were made and stained in the usual manner.

### EXPERIMENTAL OBSERVATIONS

In this series eighteen dogs were studied (Table 1) and the protocols of eight of this number are detailed below. All types of red marrow spread, reserve hemoglobin storage and hemoglobin production are included in this group of dogs. The first eight dogs given in Table 1 are included in Tables 2 and 3. Their individual histories and com-

plete autopsy protocols are given below in the same order as they appear in the tables.

The arrangement of these eight dogs in Tables 1, 2 and 3 follows the amount of red marrow spread, the first dog showing a maximal spread and the eighth dog (Dog 18-114) showing marrow exhaustion or a minimal red marrow spread.

TABLE 1  
*Approximate Percentage of Red Marrow Spread in Long Bones in Anemic Dogs*

Dog No.	Femur		Humerus		Tibia		Radius Ulna		Duration of anemia period
	Cells	Fat	Cells	Fat	Cells	Fat	Cells	Fat	
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
25-24	100	0	100	0	100	0	100	0	<i>mos.</i>
27-231	98	2	98	2	98	2	98	2	22
20-104	95	5	100	0	65	35	50	50	16
20-1	90	10	95	5	20	80	20	80	58
21-23	75	25	90	10	15	85	20	80	69
24-42	95	5	95	5	10	90	10	90	60
24-2	10	90	20	80	5	95	2	98	15
18-114	5	95	5	95	0	100	0	100	30
24-70*	98	2	100	0	75	25	65	35	88
24-23	98	2	98	2	60	40	60	40	16
20-103	95	5	98	2	60	40	25	75	5½
21-67*	95	5	95	5	40	60	20	80	51
24-89	90	10	80	20	70	30	15	85	57
24-46*	80	20	80	20	15	85	10	90	36
19-104	75	25	80	20	10	90	5	95	34
24-22	75	25	80	20	80	20	10	90	96
24-25	50	50	60	40	85	15	95	5	48
24-49	50	50	60	40	85	15	95	5	64
									44

\* Splenectomy.

Table 1 in the second half shows the marrow spread of ten dogs of similar type whose histories are not given below. Of this series two dogs showed interrupted anemia periods in the early part of their experimental histories. Dog 20-103 showed interrupted anemia periods for 3 years and continuous anemia for 15 months. Dog 19-104 showed interrupted anemia periods of 52 months and continuous anemia for 44 months. The other anemia periods were continuous.

Table 1 shows that there is no relationship between red marrow spread and the duration of the anemia period. If there is any difference the dogs of the longest anemia periods seem to show less red marrow spread but advancing years may be responsible rather than

TABLE 2

*Comparison of Red Marrow Spread and Standard Hemoglobin Production in Anemic Dogs*

Dog No.	Red marrow spread	Hemoglobin production on standard diets
25-24	Maximal—100 per cent	Sl. subnormal
27-231	Maximal—98 per cent	Subnormal
20-104	Average—85 per cent	Sl. subnormal
20-1	Average—75 per cent	Normal
21-23	Subnormal—65 per cent	Above normal
24-42	Subnormal—65 per cent	Sl. subnormal
24-2	Minimal—20 per cent	Normal
18-114	Aplasia—10 per cent	Subnormal

TABLE 3

*Spleen Metaplasia and Anemia Duration—Reserve Hemoglobin*

Dog No.	Megakaryocytes in spleen	Duration of anemia period	Theoretical reserve storage of hemoglobin at time of death
25-24	4+	1 yr., 10 mos.	Maximal
27-231	3+	1 yr., 4 mos.	Minimal
20-104	2+	4 yrs., 10 mos.	Moderate
20-1	1+	5 yrs., 9 mos.	Minimal
21-23	5+	5 yrs., 0 mos.	Low
24-42	1+	1 yr., 3 mos.	Low
24-2	2+	2 yrs., 6 mos.	Moderate
18-114	10+	7 yrs., 4 mos.	Low

the continued anemia, as we observe the same tendency in old non-anemic dogs.

Three dogs (24-70, 21-67, 24-46) were splenectomized but we have observed no differences between these dogs and the non-splenectomized group.

Table 2 indicates the extent of the red marrow spread from a maximum of 100 per cent to a minimum estimated at approximately 10 per cent. The dogs with 98 to 100 per cent red marrow spread show cellular marrow and practically no fat in ribs, vertebrae and long bones (see Figs. 1 and 2).

Table 2 also lists the general reaction of these dogs to the standard test diets—for example liver, kidney, iron and bread. It is obvious that the widest red marrow spread does not mean the most active hemoglobin production on standard diets, nor is the converse true.

Table 3 indicates that the spleen shows some marrow metaplasia with probable contribution of some marrow elements to the anemic circulation. The conspicuous feature of the spleen sections (Fig. 6) is the megakaryocyte, but nucleated red cells are sometimes numerous in the spleen pulp. We list the megakaryocytes from a minimum of 1+ to the maximal value of 10+, a purely arbitrary scale. The spleen showing the largest number of megakaryocytes is photographed (Fig. 6, Dog 18-114, Table 3). Except for the single dog 18-114 which showed some evidence of red marrow exhaustion and maximal megakaryocyte response in the spleen there is no correlation between the length of anemia period and the number of megakaryocytes in the spleen. Nor is there any correlation between the red marrow spread and the number of megakaryocytes in the spleen (Tables 2 and 3).

Table 3 also shows the theoretical reserve storage of hemoglobin or parent hemoglobin substances in each dog at time of death. This depends upon the diet intake of the last 2 or 3 weeks preceding death. It will be seen from the autopsy protocols below that there is no evidence that this reserve hemoglobin storage is to be found in the form of red cells in the bone marrow.

*Dog 25-24.*—A young adult, male bull, weight 10.02 kg. at the beginning of the anemia period May 21, 1926. Anemia was uninterrupted until time of death March 21, 1928. The blood hemoglobin level was thus kept at a level of from 40 to 50 per cent for a period of 1 year and 10 months. Death was accidental, the dog dying 3 hours after being badly bitten by another dog. The diet for the 10 week period preceding death was as follows. The theoretical reserve hemoglobin storage on this diet should be at a maximum level.

Dog 25-24. Diet, daily intake	Blood Hb. level	Hb. removed bled	Wks. before death
	<i>per cent</i>	<i>gm.</i>	
Ferric chloride (Fe 12.6 mg.), bread 400 gm., salmon 100 gm. ....	51	9.5	10
Ferric chloride (Fe 12.6 mg.), bread 400 gm., salmon 100 gm. ....	44	9.8	9
Ferric chloride (Fe 12.6 mg.), bread 400 gm., salmon 100 gm. ....	49	0	8
Ferric chloride (Fe 13 mg.), liver ash (500 gm. equiv.), bread 400 gm., salmon 100 gm. ....	59	15.0	7
Ferric chloride (Fe 13 mg.), liver ash (500 gm. equiv.), bread 400 gm., salmon 100 gm. ....	57	14.5	6
Bread 400 gm., salmon 75 gm., Klim 25 gm. ....	62	10.6	5
Ferric chloride (Fe 120 mg.), bread 400 gm., salmon 100 gm. ....	58	12.7	4
Ferric chloride (Fe 120 mg.), bread 400 gm., salmon 100 gm. ....	63	13.0	3
Ferric chloride (Fe 300 mg.), liver 300 gm., bread 300 gm. ....	63	40.3	2
Ferric chloride (Fe 300 mg.), liver 300 gm., bread 300 gm. ....	71	Death	1

*Autopsy.*—Weight of dog 13.6 kg. The animal is well developed and well nourished. The soft tissues of both fore legs are badly torn, crushed and lacerated. The skeletal muscle is in general highly colored. Heart—moderately dilated, myocardium of dark red color. Lungs are normal. In the gastrointestinal tract muscle coats there is noted the usual maple sugar or buff coloring. There is a purple-red, velvety mucosa in the duodenum and jejunum. Liver is normal except for marked congestion. Pancreas normal. Kidneys are congested, otherwise normal. The capsule strips easily leaving a smooth surface without scars. *The marrow of all the long bones* is of uniform dark red color throughout their entire length. There is no evidence of remaining fat.

#### *Microscopical Sections.*—

In the *splenic* section moderate numbers of megakaryocytes are observed. Normoblasts in clumps are scattered throughout the section in fairly large numbers. There are a few myelocytes and obviously definite myeloid metaplasia. The lymph follicles are not abnormal.

A section from the upper portion of *small intestine* shows intense congestion at tips of villi but no actual hemorrhage.

In the *liver* there is congestion about the central veins with atrophy of some of the liver cells. The cells about the central zone contain a large amount of finely granular yellowish pigment which gives no stain for iron.

The *kidneys* show normal glomeruli. Very occasional small clusters of lymphocytes are noted in the cortex. The tubular epithelial cells contain large amounts of coarsely granular yellow pigment which gives no stain for iron.

Lymph gland of mesentery shows no myeloid metaplasia and no megakaryo-

cytes. There are numerous eosinophiles. There is abundant finely granular pigment in the medulla.

*Long Bones.*—The marrow picture is one of extreme hyperplasia, there being a solid mass of cells representing all stages in development of both white and red cell series. Active marrow has entirely replaced the fat, and not a single fat cell can be seen in any of the sections from the long bones. The sections all are essentially similar, the most striking feature of which is the marked erythroid activity (Figs. 1 and 2). Throughout all the sections great numbers of normoblasts are noted as well as large numbers of their precursors. The megakaryocytes are not as numerous as seen in some specimens of extreme hyperplasia, but several are seen in each low power field. The myeloid activity is somewhat masked by the extreme erythroid hyperplasia. Neutrophilic myelocytes are much more numerous than other cells of this series. Mature red cells are present in moderate numbers in all parts of the red marrow.

*Rib and Vertebral Marrow.*—After decalcification the staining in these sections is poor. The marrow spaces however can be seen to be densely packed with cells with not a single fat cell being present. Cell types cannot be distinguished with any certainty. The mature red cells in this dog's marrow are no more numerous than in Dog 24-42 where the theoretical reserve storage of hemoglobin factors was at a low level.

*Dog 27-231.*—A young adult, female bull, weight 12.05 kg. at the beginning of the anemia period March 14, 1929. Dog has been raised since weaning on a diet of standard salmon bread. There was uninterrupted anemia until death July 22, 1930. The blood hemoglobin level was kept at a level of 40 to 50 per cent for 1 year and 4 months. Death followed an intravenous injection of ferric ammonium citrate. There were no untoward symptoms for 7 hours following the injection. The next morning there was an onset of convulsions and the animal died of respiratory failure 24 hours after the injection. The diet for the 9 week period preceding death was as follows. The theoretical reserve hemoglobin storage on this diet should be at a minimal level.

Dog 27-231. Diet, daily intake	Blood Hb. level	Hb. removed bled	Wks. before death
	<i>per cent</i>	<i>gm.</i>	
Fe 4.2 mg. intraven., bread 300 gm., salmon 100 gm., Klim 25 gm. ....	46	0	9
Fe 4.2 mg. intraven., bread 300 gm., salmon 100 gm., Klim 25 gm. ....	36	10.2	8
Bread 275 gm., salmon 100 gm., Klim 30 gm. ....	45	0	7
Bread 275 gm., salmon 100 gm., Klim 30 gm. ....	39	0	6
Fe 8.0 mg., bread 275 gm., salmon 100 gm., Klim 30 gm. ....	47	21.4	5
Fe 8.0 mg., bread 275 gm., salmon 100 gm., Klim 30 gm. ....	45	10.3	4
Bread 275 gm., salmon 100 gm., Klim 30 gm. ....	44	14.5	3
Bread 275 gm., salmon 100 gm., Klim 30 gm. ....	42	13.9	2
Bread 275 gm., salmon 100 gm., Klim 30 gm. ....	43	0	1



*Autopsy.*—The autopsy is done immediately following death. At this time the dog weighs 12.5 kg. There are no external abnormalities, the dog being well developed and well nourished. In the peritoneal cavity there are 150 cc. of a blood tinged fluid. The heart is normal in size, the valves showing no abnormalities. The lungs are pale showing minimal markings with anthracotic pigment. The stomach is contracted and contains a small amount of coffee colored fluid. There is marked congestion of the vessels of the small intestines and colon, but no definite areas of hemorrhage. The spleen, pancreas and liver appear normal. No evidence of any renal damage, the capsules stripping readily leaving a smooth unscarred surface.

*Bones.*—In the femora and humeri, there is marked hyperplasia, strawberry red throughout. The bony trabeculae are conspicuous, much more so than usual so that the entire marrow cavity feels hard. The remainder of the long bones show the same type of hyperplasia, no fat being visible.

*Microscopical Sections.*—

In the *spleen* are found many megakaryocytes. Some phagocytes containing granular yellowish brown pigment are seen. Few if any myelocytes are seen. The section also shows nucleated red cells in moderate numbers.

The *liver* shows a widespread portal necrosis. The greater number of liver cells show some injury with fatty changes. A few polymorphonuclears are scattered throughout the parenchyma. This change is obviously related to the injection of iron citrate.

*Bone Marrow.*—Sections from all the large bones are essentially similar. All of the fat has been demobilized and replaced by active marrow. Scarcely a fat cell can be found. The normoblasts and their parent cells are present in enormous numbers. There are no large sinuses packed with red cells but the latter are distributed diffusely throughout the marrow. The megakaryocytes are not quite as numerous as usually seen in marrow showing as much hyperplasia as this. They show occasional phagocytosis of cell fragments. Eosinophilic myelocytes are few in number.

No fat cells are present in sections from the *vertebrae* and *ribs*. The hyperplastic marrow shows great erythroid activity as enormous numbers of normoblasts and large numbers of their precursors are present.

*Dog 20-104.*—An adult, male bull, weight 8.0 kg. at the beginning of the anemia period July 15, 1920. Until 1923 the anemia periods were interrupted and the blood hemoglobin level somewhat higher than the usual level of 40 to 50 per cent. From 1923 until time of death May 22, 1925, the blood hemoglobin was kept at the usual level and the anemia was uninterrupted. The animal was sacrificed by *perfusion* and the muscle extracted. The diet for the 10 week period preceding death was as follows. The theoretical reserve hemoglobin storage on this diet should be at a moderate level.

Dog 20-104. Diet, daily intake	Blood Hb. level	Hb. removed bled	Wks. before death
	per cent	gm.	
Pig brain 200 gm., bread 300 gm. ....	43	0	10
Pig kidney 200 gm., bread 300 gm. ....	55	12.8	9
Beef muscle 50 gm., bread 400 gm. ....	50	0	8
Pig muscle 200 gm., bread 300 gm. ....	56	10.6	7
Pig muscle 200 gm., bread 300 gm. ....	47	0	6
Bread 400 gm., salmon 75 gm. ....	44	0	5
Pig heart 200 gm., bread 300 gm. ....	48	9.9	4
Pig heart 200 gm., bread 300 gm. ....	48	12.4	3
Bread 350 gm., salmon 100 gm. ....	40	0	2
Pig liver 150 gm., bread 300 gm. ....	57	15.7	1

*Autopsy.*—Weight of dog 10.5 kg. The animal is well nourished and there are no external abnormalities. Subcutaneous tissue is pale as are also the heart, lungs, spleen, pancreas and liver. The kidneys are particularly pale in region of the pyramids. Lymph nodes contain brilliant vital red dye, some of which is also seen in the renal cortical region.

*Bones.*—In the femora and humeri the marrow is uniformly dark red throughout. The tibiae, radii and ulnae have marrow of the same appearance in the upper two-thirds, but the distal portions are filled with pale fat.

*Microscopical Sections.*—

Because of the perfusion very few red cells are to be found in any of the tissues.

The *liver* contains considerable glycogen. In the tubular cells of the *kidneys* there is a small amount of yellow pigment which is non-iron staining.

In the *spleen* there are found scattered normoblasts in moderate numbers. Megaloblasts are not conspicuous but several are seen. Very few pigment containing phagocytes are present.

*Bone Marrow.*—Advanced hyperplasia with only an occasional isolated fat cell visible in the long bones. Normoblasts are present in extremely large numbers. Red cells are few because of the perfusion. Capillaries are infrequently noted and no sinuses densely packed with red cells are observed. Red cells in small numbers are scattered about in the stroma.

The cells of the granulocytic series are also numerous, several eosinophilic myelocytes and early leucocytes being present in a medium high power field. The megakaryocytes are present in numbers usually found in such hyperplastic marrow.

*Vertebrae.*—No fat. Few megakaryocytes can be distinguished. Normoblasts are the only cells which can be differentiated. These are present in large numbers.

*Dog 20-1.*—A young adult, female bull, weight 13.3 kg. at the beginning of the anemia period October 27, 1919. The anemia period was interrupted during the

summer months for 3 years. From October 10, 1923, until June 24, 1925, there was continuous anemia with the blood hemoglobin level being maintained at the usual level of 40 to 50 per cent. The dog was sacrificed and perfused for extraction of muscle hemoglobin. The diet for the 12 week period preceding death is given in following table. The theoretical reserve hemoglobin storage on this diet should be at a minimal level.

Dog 20-1. Diet, daily intake	Blood Hb. level	Hb. removed bled	Wks. before death
	per cent	gm.	
Prunes 300 gm., bread 400 gm. ....	58	23.3	12
Prunes 300 gm., bread 400 gm. ....	56	19.2	11
Bread 500 gm. ....	45	19.0	10
Bread 500 gm., salmon 50 gm. ....	46	0	9
Butter 50 gm., bread 400 gm. ....	44	11.1	8
Butter 50 gm., bread 400 gm. ....	48	11.6	7
Bread 500 gm., salmon 50 gm. ....	42	0	6
Bread 500 gm., salmon 50 gm. ....	48	0	5
Fasting.....	42	12.4	4
Fasting.....	47	0	3
Bread 500 gm., salmon 50 gm. ....	49	0	2
Bread 500 gm., salmon 50 gm. ....	?	13.9	1

*Autopsy.*—The weight of the dog is 18.8 kg. The animal is exceptionally well developed and well nourished. There is a large amount of subcutaneous fat which is stained with the brilliant vital red dye. The muscles appear to contain somewhat more muscle hemoglobin than in other anemic dogs. The heart, lungs, spleen, pancreas, liver and neck organs are normal. The gall bladder shows evidence of chronic inflammation. The staining with the vital dye is also noted in mesenteric glands, ovaries, adrenals, uterus, intestines, bladder wall and kidneys. Kidneys show a few scars.

The *marrow* as seen in the femora and humeri is extremely cellular with small grayish islands. The upper ends of the tibiae and radii are dark red; the middle portions contain considerable fat and the lower ends are all fat. The ulnae show red marrow throughout. Ribs and vertebrae not recorded.

*Microscopical Sections.*—

In the *spleen* the normal structures are interspersed by small groups of normoblasts at frequent intervals but their precursors are difficult to distinguish with any certainty. There are few megakaryocytes.

The *liver* cells show no abnormalities. The *gall bladder* shows infiltration with mononuclear cells in the submucosa.

The *kidneys* show occasional small clusters of round cells in the cortex near the surface. An occasional glomerulus shows some injury with thickening of

the capsule. The epithelial cells of the convoluted tubules are heavily loaded with a yellow granular pigment.

*Femora*.—This hyperplastic marrow has a loose, coarse textured arrangement of the cells following the perfusion. Relatively few mature red cells are seen. The normoblasts are present in fairly large numbers. Several megaloblasts, mainly in small clumps, are observed. Cells of the granulocytic series are represented by neutrophilic myelocytes although an occasional mature leucocyte can be seen. The megakaryocytes are present in numbers usually consistent with hyperplastic marrow of this grade.

*Humeri*.—Except for a smaller amount of residual fat the marrow is similar to that of the femora.

*Tibiae*.—The proportion of fat to active marrow is approximately reversed from what is seen in the femora and humeri. Like that marrow the sinuses and large vessels are washed free from red cells by the perfusion.

*Ulnae*.—Essentially similar to the tibial marrow.

*Dog 21-23*.—A young adult, bull mongrel, weight 14.1 kg. at the beginning of the anemia period in May, 1921. There were interrupted periods of anemia until September 3, 1924, when from this date until death June 5, 1926, there was an uninterrupted anemia with a blood hemoglobin level of from 40 to 50 per cent. The dog died immediately following an intravenous injection of hemoglobin on June 5, 1926. The diet period for 11 weeks preceding death was as follows. The theoretical reserve hemoglobin storage on this diet should be at a low level.

Dog 21-23. Diet, daily intake	Blood Hb. level	Hb. removed bled	Wks. before death
	per cent	gm.	
Liver 100 gm., bread 250 gm. ....	73	43.4	12
Liver 100 gm., bread 250 gm. ....	54	55.5	11
Bread 400 gm., salmon 50 gm. ....	48	13.6	10
Bread 400 gm., salmon 50 gm. ....	55	17.6	9
Bread 400 gm., salmon 50 gm. ....	46	25.2	8
Bread 400 gm., salmon 50 gm. ....	32	9.3	7
Muscle Hb. intraven., bread 325 gm., salmon 75 gm.	36	0	6
Muscle Hb. intraven., bread 325 gm., salmon 75 gm.	28	0	5
Bread 350 gm., salmon 50 gm. ....	38	0	4
Bread 350 gm., salmon 50 gm. ....	46	8.2	3
Bread 350 gm., salmon 50 gm. ....	47	0	2
Blood Hb. intraven. (4 doses 2.04 gm. total). ....	Death		1

*Autopsy*.—Weight of dog 17.2 kg. The animal is well developed and well nourished with no external abnormalities. There is apparent slow and incomplete coagulation, the right ventricle being greatly dilated and containing a few soft clots. Moderate anthracotic pigment is present in lungs. The liver is con-

gested and the spleen is congested and enlarged having conspicuous malpighian bodies which show through the capsule. Pancreas is normal. The kidney architecture is undisturbed, there being some congestion and a few small scars. The muscle coats of the intestinal tract show a pale buff color. There is only moderate congestion of the mucosa. The mesenteric glands are pinkish (vital red dye).

*Bones.*—

Femora—*distal* one-third almost entirely brick red marrow. The middle one-third is at least one-half fat.

Humeri—cellular, brick red throughout.

Tibiae—upper one-half consists of more than 60 per cent fat, the lower is almost all fat.

Radii—upper one-half is red, but contains large amounts of fat. The lower one-half is largely fat, less than one-fifth showing red marrow.

Ulnae—similar to marrow found in radius.

*Microscopical Sections.*—

The *spleen* shows numerous megakaryocytes, several being seen in each high power field. No myelocytes made out. There are scattered clusters of normoblasts. The malpighian bodies and germinal centers are conspicuous.

Mesenteric lymph gland shows some fine granular pigment in its medulla. No evidence of marrow cells.

The *liver* shows a moderate amount of fat which is centrally located. The liver cells are frothy looking and contain no pigment.

The *kidneys* give evidence of degenerative change in some glomerular tufts. There is no increase in stroma. Casts are absent. The tubular epithelial cells contain considerable yellow pigment.

*Femora.*—Large solid cellular areas of active marrow alternate with almost as large areas of fat. Normoblasts are seen in fairly large numbers but are not as numerous as frequently seen in marrow showing as much hyperplasia as this. Megaloblastic clumps are observed. Mature red cells are not numerous. The cellular mass is not loosely arranged with a large amount of intercellular matrix, but is extremely compact and densely packed. There are no large blood filled sinuses. Mitotic figures are not uncommon. Megakaryocytes average 2 to 3 per medium high power field. A fair number of myelocytes and metamyelocytes are observed. There are large numbers of undifferentiated cells.

*Tibiae, Radii and Ulnae.*—Section shows about 85 per cent fat. The areas of active marrow are apparently no different from those of the femora.

*Humeri.*—A highly compact and complex cellular mass with a small scattering of fat cells. There is no apparent difference from the femoral marrow. Large or relatively large numbers of megaloblasts are observed.

*Ribs.*—Cells are hard to differentiate except for the numerous megakaryocytes and normoblasts. Only an occasional fat cell is visible.

*Dog. 24-42.*—A young adult, male bull, weight 15.6 kg. at the beginning of the

anemia period September 3, 1924. There was uninterrupted anemia until death December 3, 1925. The blood hemoglobin level during this interval was kept depressed to 40 to 50 per cent by suitable bleedings. Death followed within a few hours after the onset of an acute respiratory infection. The diet for the 10 week period preceding death was as follows. On this diet the theoretical reserve hemoglobin storage should have been low.

Dog 24-42. Diet, daily intake	Blood Hb. level	Hb. removed bled	Wks. before death
	per cent	gm.	
Cooked dried apricots 250 gm., bread 450 gm.	58	13.4	10
Cooked dried apricots 250 gm., bread 350 gm.	50	17.9	9
Bread 600 gm.	47	0	8
Bread 600 gm.	48	0	7
Fresh cooked apples 200 gm., bread 400 gm.	44	12.0	6
Fresh cooked apples 200 gm., bread 400 gm.	48	0	5
Bread 600 gm.	48	13.0	4
Bread 600 gm.	47	0	3
Bone marrow powder 30 gm., bread 650 gm.	41	10.5	2
Bone marrow powder 30 gm., bread 500 gm., salmon 50 gm.	39	0	1

*Autopsy.*—The weight of the dog is 15.2 kg. The dog is well developed and well nourished having no external abnormalities. Heart is normal. There is an acute pleurisy, the pleural surfaces being speckled with numerous small hemorrhages. Edema and congestion of lung is marked and on cut section a heavy frothy exudate can be pressed from the alveoli. The spleen and pancreas are normal. The intestinal tract is stained a dark brown, the mucosa being pink and injected, with evidence of a slight inflammation in the jejunum. The liver is slightly fatty, a frozen section of which shows extensive focal necroses with many leucocytes. The parenchyma of each kidney is normal. In the pelvis of the left kidney there are moderate sized calculi and in the right two smaller ones.

*Bones.*—

Femora—brick red with fat in central core.

Humeri—brick red throughout.

Tibiae—upper two-fifths red, middle one-fifth mottled red and fat, lower two-fifths pure fat.

Radii—upper two-thirds brick red, pale in lower one-third.

Ulnae—upper one-third mottled with some fat, lower two-thirds pure fat.

*Microscopical Sections.*—

The sections of the lungs show small areas where the alveoli are densely packed with red cells and polymorphonuclear leucocytes. In some of these areas there

is early organization. Several of the alveoli contain delicate strands of fibrin a few polymorphonuclears and some large mononuclears. There is considerable edema.

*Spleen* shows small clusters of normoblasts. The megakaryocytes average less than one in each high dry field. The malpighian bodies are prominent and they have large germinal centers. In the latter are found many mitotic figures. There is extreme *liver* injury and but relatively few normal appearing liver cells remain, these being located about the portal region. The cells about the central zone show extensive necrosis and fatty changes with infiltration of large numbers of polymorphonuclears. The hyalin necrosis involves approximately one-third of the liver parenchyma.

The *kidneys* appear normal except for the cells of the convoluted tubules which contain a large amount of yellow pigment.

*Femora*.—A large amount of hyperplastic marrow which shows only occasional fat cells. The normoblasts are seen in extremely large numbers and are generally scattered throughout the section. They are the most conspicuous single cell type. Normal red cells are numerous. Megaloblasts are also numerous and can be seen in clumps made up of several adherent cells. The red cell chain is as conspicuous if not more so than in Dog 25-24 in which dog is recorded a theoretical maximum hemoglobin reserve. Megakaryocytes are large with brilliant pink cytoplasm. They are present in large numbers, sometimes several being seen in a medium high power field. Some megakaryocytes are phagocytic. The granulocytes are somewhat overshadowed by the great activity in the red cell series. Myelocytes of the eosinophilic and neutrophilic types are noted in fair numbers.

*Humeri*.—Almost solid areas of hyperplasia. Few scattered fat cells and an occasional area of solid fat is noted. Cellular picture essentially similar to the femora.

*Tibiae*.—No essential difference noted in the sections from the upper and middle thirds. The greater part of the marrow consists of fat; where there is some hyperplasia, however, there is evidence of great activity in the erythroid series. Normoblasts are exceptionally numerous.

*Dog 24-2*.—Adult, male white bull, weight 16.8 kg. Dog was anemic from November 21, 1923, to March 12, 1924, when the dog developed distemper. He recovered and was put in the stock colony until July 21, 1927, when he was again made anemic and remained at the usual anemia level until death January 10, 1930. This dog at times had albuminuria and occasional casts but the extent of his nephritis was not appreciated. Dog was killed with ether. On the diet listed below during 11 weeks preceding death, the theoretical reserve hemoglobin storage was at a moderate level, neither high nor very low.

Dog 24-2. Diet, daily intake	Blood Hb. level	Hb. removed bled	Wks. before death
	<i>per cent</i>	<i>gm.</i>	
Bread 350 gm., salmon 75 gm., Klim 25 gm. ....	49	19.7	11
Bread 350 gm., salmon 75 gm., Klim 25 gm. ....	57	13.9	10
Bread 350 gm., salmon 75 gm., Klim 25 gm. ....	45	1.2	9
Apricots 200 gm., bread 300 gm., salmon 100 gm., Klim 30 gm. ....	57	19.3	8
Apricots 200 gm., bread 300 gm., salmon 100 gm., Klim 30 gm.. ....	55	17.5	7
Bread 325 gm., salmon 100 gm., Klim 40 gm. ....	48	1.4	6
Bread 325 gm., salmon 100 gm., Klim 40 gm. ....	50	23.8	5
Bread 325 gm., salmon 100 gm., Klim 40 gm. ....	45	1.3	4
Liver 100 gm., bread 200 gm., Klim 40 gm. ....	43	40.2	3
Liver 100 gm., bread 200 gm., Klim 40 gm. ....	43	1.3	2
Bread 275 gm., salmon 125 gm., Klim 35 gm. ....	50	15.7	1

*Autopsy.*—Weight of dog at death 12.5 kg. Dog is slightly emaciated but the body shows nothing unusual. Heart shows normal valves and muscle tissue. In the left auricle above the mitral valve is a granular vegetation about 1 x 3 mm. It is calcified and obviously old, perhaps related to the attack of distemper 6 years previously. Lungs normal. Spleen normal. Liver, gall bladder, pancreas and gastrointestinal tract are perfectly normal in gross. Kidneys show relatively little gross change but the striae of the cortex are irregular. Bladder urine shows albumin, casts and a few red and white cells.

*Bones.*—

Femora contain mostly fat in the shaft and cancellous tissue.

Humeri contain fat in the upper half and some red marrow in the lower half.

Tibiae almost all fat in shaft and cancellous bone.

Radii and ulnae as in tibiae.

The ribs and vertebrae show red marrow which in gross appears as usual.

*Microscopical Sections.*—

*Heart.*—The muscle fibres are normal in most areas. The vegetations noted in gross are in most instances old and organized and covered by intact endothelium. A few fresh vegetations are found and below these areas polymorphonuclears are numerous—a chronic endocarditis with an acute exacerbation.

*Kidneys.*—As might be expected from the endocarditis kidneys show much glomerular injury with adhesions between glomerular tufts and capsule. The capsules are thick and fibrous. There are nests of mononuclear cells scattered throughout the cortex. Many tubules are dilated and some contain casts. The blood vessels are thickened. We have the picture of a primary nephritis as seen in man. Many of the convoluted tubules show a coarsely granular bright yellow



pigment within the epithelium. This pigment does not give an iron stain.

*Spleen* shows nothing unusual. Megakaryocytes are not numerous but when found appear in small groups. Nucleated red cells are very infrequent. Eosinophilic cells are numerous. Malpighian bodies normal. The arterioles show hyalin changes and much thickening.

*Liver* shows normal epithelium. The liver cells in the centers of the lobules show some finely granular yellow pigment which is negative for iron. No abnormal cells found anywhere.

Pancreas and gastrointestinal tract normal.

*Thyroid* shows an adenoma made up of small acini and containing no colloid—fetal adenoma. Elsewhere thyroid acini are normal.

*Bones*.—Femora and humeri show marrow cavities filled with fat. Only small areas close to the bony shell show the usual red marrow. Where this red marrow is found it is normal in all respects and obviously made up of active cells. Nucleated red cells are very numerous. Megakaryocytes are numerous and large and myelocytes are present as usual.

Tibiae show practically all fat with only an occasional cluster of normal marrow cells.

Vertebrae show a good deal of red marrow but one-half to four-fifths of these sections are made up of normal fat cells. Where the red marrow is found all of its cells appear normal and very active. Nucleated red cells are very numerous.

*Rib*.—This tissue shows more red marrow hyperplasia than any part of the skeleton but even here fat cells are seen in all sections. The marrow cells are all normal and obviously active. Nucleated red cells are found everywhere.

*Dog 18-114*.—A young adult, female bull mongrel, weight 15.4 kg. at the beginning of the anemia period February 12, 1920. The anemia periods alternated with recovery periods during 3 years. From February, 1923, until June, 1927, there was continuous anemia with the usual hemoglobin level of 40 to 50 per cent. Just before death there was vomiting and drowsiness with a temperature of 98.7°F. There was an extremely foul breath. On the day before death there was a non-protein nitrogen of 231 mg. per 100 cc. and a blood urea nitrogen of 146 mg. per 100 cc. The urine contained albumin, Esbach 1.5 gm./liter, and many granular and epithelial casts, also a few red cells and leucocytes. The dog was sacrificed with ether and bleeding on June 16, 1927. The diet for the 7 week period preceding death is given in the following table, except that liver residue was only given three times in the last week. The theoretical reserve hemoglobin storage on this diet was at a low level.

Dog 18-114. Diet, daily intake	Blood Hb. level	Hb. removed bled	Wks. before death
	per cent	gm.	
Liver extract equiv. 600 gm. liver, bread 250 gm., salmon 75 gm. ....	50	10.5	7
Liver extract equiv. 600 gm. liver, bread 250 gm., salmon 75 gm. ....	46	0	6
Bread 250 gm., salmon 100 gm., Klim 50 gm. ....	47	0	5
Bread 225 gm., salmon 75 gm., Klim 25 gm. ....	48	14.7	4
Bread 225 gm., salmon 75 gm., Klim 25 gm. ....	44	12.7	3
Bread 225 gm., salmon 75 gm., Klim 25 gm. ....	48	0	2
Liver residue equiv. 600 gm. liver, bread 250 gm., Klim 25 gm. ....	49	0	1

*Autopsy.*—June 16, 1927. The weight of the dog is 12.7 kg. There has been considerable weight loss but no other abnormalities are noted. The heart shows hypertrophy of the left ventricle but no valvular changes. Lungs are collapsed and heavily pigmented. The spleen, liver, gall bladder and urinary bladder are normal. The intestines show the maple sugar color in the muscle coat which follows prolonged liver feeding. There is a chronic pancreatitis which is more marked in the middle third. The adrenals are enlarged showing over 50 per cent increase in size. Both *kidneys* are enlarged and firm with evident increase in connective tissue, no hemorrhages, no irregularity of the architecture. The capsule strips easily leaving a smooth unscarred surface. The pyramids are pale, the pelves normal.

*Bones.*—All the bones are more brittle than those of younger dogs.

*Femora.* Upper end and neck pale brick red, lower end all white. Middle three-fifths nearly all fat. The marrow is watery and edematous differing from the usual anemia marrow.

*Humeri.* Upper and pinkish red cancellous marrow. The lower end is all white cancellous bone. The middle three-fifths is mostly fat with marginal patches of red marrow.

*Tibiae, radii and ulnae.* No red marrow to be seen. Shaft contains fat only. Epiphyseal ends are white.

The vertebral and rib marrow has the normal dark red color.

#### *Microscopical Sections.*—

The heart shows scattered small collections of polymorphonuclear and mononuclear cells indicating focal injury of the myocardium. Often these areas are adjacent to small blood vessels. The muscular fibres are moderately hypertrophied.

The *spleen* shows evidence of definite *erythropoiesis*. Large numbers of megakaryoblasts and even greater numbers of normoblasts are evident. Megakaryocytes are seen in larger numbers than in any spleen of this series (Fig. 6). A moderate

pigment within the epithelium. This pigment does not give an iron stain.

*Spleen* shows nothing unusual. Megakaryocytes are not numerous but when found appear in small groups. Nucleated red cells are very infrequent. Eosinophilic cells are numerous. Malpighian bodies normal. The arterioles show hyalin changes and much thickening.

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	per cent	gm.	
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Bread 225 gm., salmon 75 gm., Klim 25 gm. ....	48	14.7	4
Bread 225 gm., salmon 75 gm., Klim 25 gm. ....	44	12.7	3
Bread 225 gm., salmon 75 gm., Klim 25 gm. ....	48	0	2
Liver residue equiv. 600 gm. liver, bread 250 gm., Klim 25 gm. ....	49	0	1

*Autopsy.*—June 16, 1927. The weight of the dog is 12.7 kg. There has been considerable weight loss but no other abnormalities are noted. The heart shows hypertrophy of the left ventricle but no valvular changes. Lungs are collapsed and heavily pigmented. The spleen, liver, gall bladder and urinary bladder are normal. The intestines show the maple sugar color in the muscle coat which follows prolonged liver feeding. There is a chronic pancreatitis which is more marked in the middle third. The adrenals are enlarged showing over 50 per cent increase in size. Both kidneys are enlarged and firm with evident increase in connective tissue, no hemorrhages, no irregularity of the architecture. The capsule strips easily leaving a smooth unscarred surface. The pyramids are pale, the pelves normal.

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The spleen shows evidence of definite erythropoiesis. Large numbers of megakaryocytes and even greater numbers of normoblasts are evident. Megakaryocytes are seen in larger numbers than in any spleen of this series (Fig. 6). A moderate

amount of yellowish brown pigment in phagocytic cells is noted throughout the pulp. The lymph follicles are of normal appearance.

In the *pancreas* small areas of acinar tissue have been replaced by fibrous tissue in which the ducts stand out prominently. The remaining pancreatic tissue is normal.

The *liver* contains a small number of scattered fat cells. Polymorphonuclears in small numbers are seen throughout the liver parenchyma.

The *kidneys* show extensive glomerular injury with adhesions and fibrosis of the tufts. There is marked thickening of the capsules and adhesions between glomerulus and capsule are numerous. Hyalin thrombi are frequently noted. The tubules are much larger than normal having a low epithelial lining. Many of the tubules contain hyalin casts, some of which are impregnated with lime salts. The cortex shows considerable interstitial fibrosis and mononuclear infiltration. The tubular epithelial cells contain a moderate amount of yellow pigment and many fat droplets.

*Right Femur.*—Two sections are available from right femur. These consist largely of fat. Small areas of active marrow are present on the peripheral portion of the marrow core. A very small amount of fat has been replaced by red marrow for the small amount of marrow activity is confined to areas *between* the fat cells. Of the cells present, normoblasts form a fairly large proportion. The megaloblasts in strands and small clumps are present in somewhat unusual numbers. Erythroblasts are also numerous. Relatively few myelocytes or early leucocytes can be seen. There are several megakaryocytes in each medium high power field (Fig. 5).

*Right Tibiae.*—Entirely made up of fat; no marrow cells.

*Left Humerus.*—This section is entirely similar to the ones from the right femur—both in the amount of active marrow and in the type of cells present (Fig. 4).

*Ribs.*—The rib marrow shows about one-third fat. Cellular types in all decalcified sections are differentiated with difficulty. Megakaryocytes are made out with ease and are present in large numbers. Cells having an eosinophilic cytoplasm are numerous.

*Vertebrae.*—Of the four vertebral sections three are practically similar to the rib sections. One of the vertebral sections, however, gives a picture of complete aplasia. The marrow is entirely made up of fat with but an occasional very small group of cells (Fig. 3).

*Note.*—This dog shows only *slight failing* in capacity to produce hemoglobin and red blood cells even in the face of extreme hypoplasia or exhaustion of red marrow.

#### DISCUSSION

In spite of the comprehensive observations of Peabody (5), Sabin (8, 3), Doan (1, 2), Cunningham (3) and others, we felt that it was essential to carry out a study of the red marrow in these dogs whose life history is known from birth. The only abnormality in most cases

is a long continued severe anemia due to bleeding. The pigment production of this type of dog has been carefully studied in this laboratory and includes both hemoglobin and bile pigment output under a variety of controlled conditions.

Expecting marrow exhaustion in these long continued anemia experiments, we were disappointed, and it is evident that the red marrow of these dogs can stand the strain of a continuous severe anemia for very long periods and in some cases throughout the entire life of the dog. In Dog 18-114 (see Table 2) there seems to be some evidence of marrow exhaustion and the red marrow spread is reduced to very restricted limits. In spite of this limited amount of red marrow this dog produced reasonable amounts of new hemoglobin from week to week and there was no spectacular fall in the output of hemoglobin and new red cells (see history above, Dog 18-114). We may say that there is a moderate drop in hemoglobin production during the last few weeks of life but it is in no way proportional to the great shrinkage in the red marrow area of spread.

Another dog shows a limited red marrow spread (Dog 24-2, Table 2 and history above) but this dog has approximately twice as much red marrow as Dog 18-114. This second dog (24-2) shows no diminution in the output of hemoglobin and red cells on standard diets in the last weeks of life. This red marrow in spite of its limited area was able to produce the usual amount of new red cells and there is no evidence to indicate marrow exhaustion although there is red marrow shrinkage.

In both these dogs with very limited red marrow spread (Dogs 24-2 and 18-114) the red marrow elements remaining appear in all respects normal and show evidence of the usual active production of red cells. It would seem that this small amount of red marrow must be producing red cells with maximum efficiency per gram of red marrow.

These same two dogs show a considerable degree of chronic nephritis and Dog 18-114 was approaching uremia. We can find no clear evidence that the chronic nephritis is related to the limited or decreased red marrow spread as we can refer to two dogs in the second half of Table 1 (Dogs 19-104 and 24-49) with similar types and grade of nephritis but with an average red marrow spread. Chronic nephritis appears in dogs past middle age and frequently in dogs which have had distemper. Along with advancing years there is a tendency for

the red marrow to occupy a smaller area of the bony skeleton. It is possible that this shrinkage of the red marrow area may be a part of the atrophy of old age in both normal and anemic dogs.

Spleen metaplasia with erythrocytogenesis in this series of dogs appears definite but not striking in the majority of cases. Among the early workers in this field Morris (4) observed in anemic rabbits a somewhat similar reaction in the spleen. He noted erythrocytogenesis in the liver as well. We have never observed any change in the livers of these dogs indicating marrow metaplasia. Also the lymph glands are free of evidences of marrow metaplasia and erythrocytogenesis.

The spleen shows varying numbers of megakaryocytes, see Table 3 and Fig. 6. There are nests of nucleated red cells in the spleen pulp in practically all cases. One might explain these nucleated red cells as coming from the marrow and not arising within the spleen but the evidence seems to indicate that some red cells are formed in the spleen pulp. We do not believe that the spleen is ever a large factor in red cell production under these conditions. An occasional myelocyte is seen in the spleen pulp.

#### SUMMARY

In long continued severe anemia due to bleeding in dogs we may observe all degrees of red marrow spread. The maximal red marrow spread takes in the total marrow area in ribs, vertebrae and long bones. The minimal red marrow spread may involve but 10 to 20 per cent of this maximal area. All gradations between these extremes are observed in this series.

The extent of the red marrow spread is not dependent upon the length of the anemia period nor is it related to the capacity of the dog to produce new hemoglobin and red cells on standard diets.

Evidence points to the liver as concerned with this hemoglobin production and liver function may set the limits for hemoglobin production on these standard diets. This in turn may determine the needful red marrow spread.

During favorable diet periods there is a storage of hemoglobin or hemoglobin precursors which come out later in the control periods as finished red cells. This reserve of hemoglobin producing material is not stored as mature red cells in the marrow or elsewhere but as

intermediates stored probably in the liver and red marrow but also perhaps in the kidney and spleen.

Red marrow very rarely gives evidence of exhaustion even after many years of continuous severe anemia (Dog 18-114). This would seem to consist of a shrinkage of the red marrow area rather than a degenerative change of the red marrow cells.

The spleen shows some evidence of erythropoiesis in these dogs. Megakaryocytes in some instances are conspicuous (Fig. 6) and nests of nucleated red cells are found in the spleen pulp but it is unlikely that the spleen contributes any large amount of red cells to the anemic circulation in these experiments.

There is no evidence that erythropoiesis occurs in the liver or lymphatic tissue in these dogs.

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#### EXPLANATION OF PLATES

The photographs were taken from sections stained with hematoxylin and eosin after fixation in Zenker's fluid.

##### PLATE 29

FIG. 1. Maximal red marrow spread and hyperplasia. Dog 25-24—radius marrow.  $\times 107$ .

FIG. 2. Maximal marrow hyperplasia. Dog 25-24—radius marrow.  $\times 580$ .

##### PLATE 30

FIG. 3. Minimal red marrow spread. Dog 18-114—body of vertebra.  $\times 107$ .

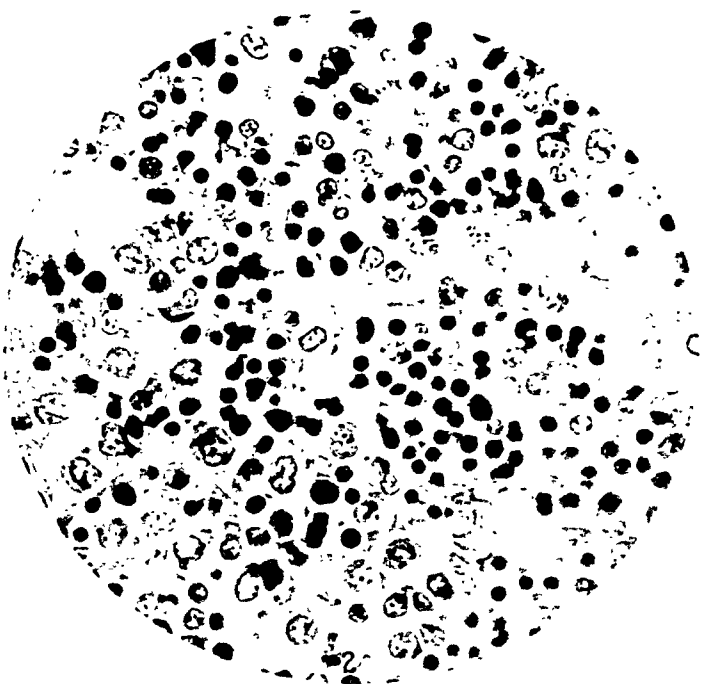
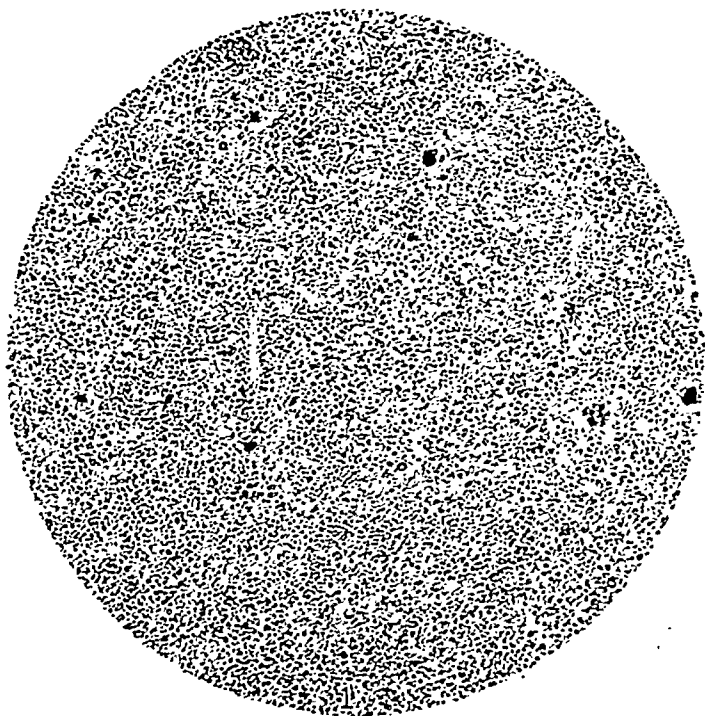
FIG. 4. Minimal red marrow spread. Dog 18-114—humerus marrow.  $\times 111$ .



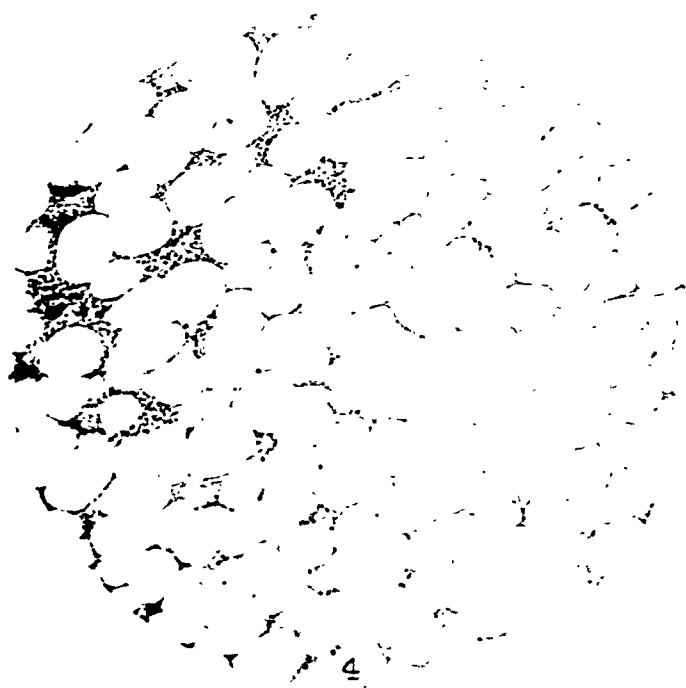
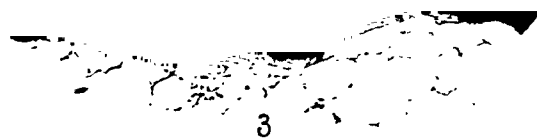
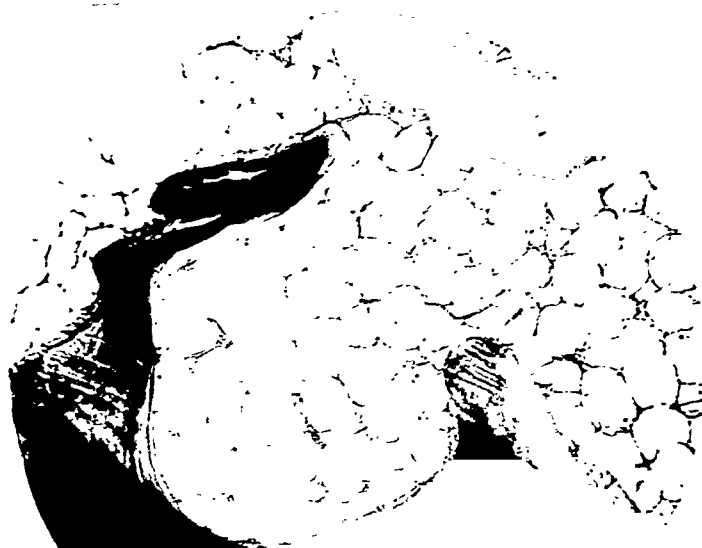
## PLATE 31

FIG. 5. Minimal red marrow spread. Dog 18-114—femur marrow.  $\times 527$ .

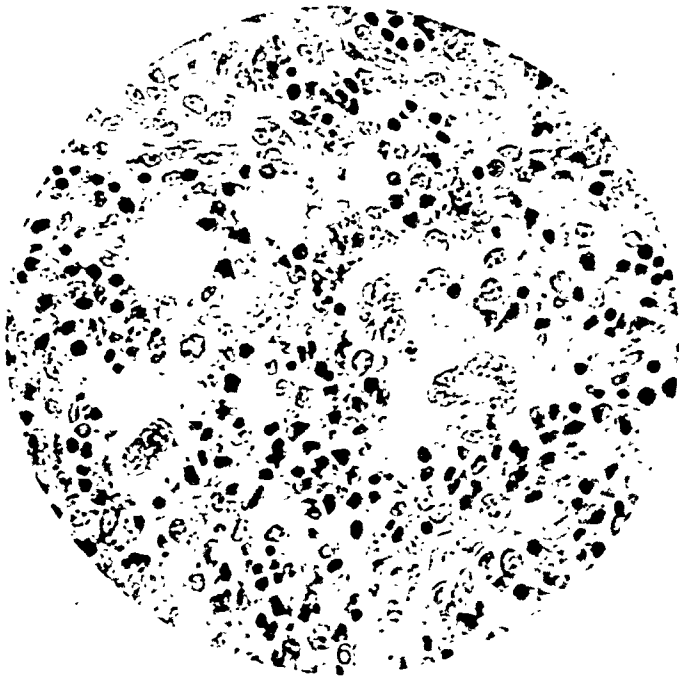
FIG. 6. Spleen erythrogenesis—megakaryocytes and nucleated red cells. Dog 18-114.  $\times 382$ .











(Osteoblasts, Mononuclear, in center.)



## THE INFLUENCE OF PLASMA PROTEIN ON THE CHLORIDE CONTENT OF THE CEREBROSPINAL FLUID

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(Received for publication, May 28, 1932)

During the past two decades much attention has been given to the mode of formation of the cerebrospinal fluid. Until recently it was generally considered that the fluid was actually secreted by cells of the choroid plexus. This theory was first suggested 70 years ago by Schmidt (11), Faivre (3), and Luschka, and it was the working basis of almost all of the numerous investigations of this subject until 1912. In that year Mestrezat (10) brought forward the theory that the cerebrospinal fluid is a dialysate in equilibrium with the blood plasma, the choroid plexus acting merely as a simple dialyzing membrane. Since 1912 many investigations have been undertaken to determine the actual mechanism, and the results of most of this work have been reviewed by Fremont-Smith (4) who, from his survey and from the results of his own researches, has been led to think that "there is no good evidence for secretion."

There are other authors, however, who are not in accord with the view of Fremont-Smith, as there are some facts that have not been easily explained by Mestrezat's theory of dialysis. The center of difficulty has chiefly been the marked disparity between the chloride content of the blood and spinal fluid. A very similar disparity has been found in several other fluids of the body, and therefore the difference seems to be a real one, of fundamental importance.

Many of the conflicting results which have been obtained for the blood and other body fluids may be explained by the inherent difficulties of tests, and by the non-specificity of certain analytical and colorimetric methods when applied to various constituents of the above fluids. However, this explanation cannot be used in the case of chlorides which can be estimated with accuracy and certainty. Levinson (8) makes the comment that "there has been greater uniformity in the results of various workers upon the chloride content of the cerebrospinal fluid than upon its other chemical constituents."



need for accuracy of this figure, 1.1 per cent was accepted for all the calculations, thus sparing the quantity of cerebrospinal fluid needed for the analyses.

## RESULTS

The results of two of the four experiments are tabulated, those of the other two experiments being quite similar.

TABLE I

Day	Residue		Water		Plasma protein	Sodium*		Disparity	Chloride*		Disparity
	Plasma	Cerebro-spinal fluid	Plasma	Cerebro-spinal fluid		Plasma	Cerebro-spinal fluid		Plasma	Cerebro-spinal fluid	
Dog 1											
1	gm. per cent	gm. per cent	gm. per cent	gm. per cent	gm. per cent	mg. per cent	mg. per cent	mg.	mg. per cent	mg. per cent	mg.
1	8.5	1.1	95.1	98.9	6.6	355	345	10	409	470	61
Plasmapheresis (350 cc. of blood withdrawn, corpuscles washed, resuspended, reinjected 14 times)											
6	4.5	1.1	95.5	98.9	2.9	345	339	6	439	466	27
7	5.5	1.1	94.5	98.9	3.9	352	348	4	436	469	33
8	6.1	1.1	93.9	98.9	4.5	352	346	4	434	468	34
10	8.3	1.1	91.7	98.9	6.6	355	349	6	432	476	44
Dog 2											
1	9.1	1.1	90.9	98.9	7.4	374	357	18	423	486	63
Plasmapheresis (400 cc. of blood withdrawn, corpuscles washed, resuspended, reinjected 7 times)											
3	5.2	1.1	94.8	98.9	3.8	342	338	4	438	468	30
4	6.2	1.1	93.8	98.9	4.7	355	351	4	437	485	48
5	7.0	1.1	93.0	98.9	5.5	361	349	12	426	474	48
7	7.9	1.1	92.1	98.9	6.4	368	353	15	420	484	64

\* Corrected for the water content of the plasma and cerebrospinal fluid.

In both experiments it will be seen at once that there is a very striking decrease in the disparity of the chloride concentrations of the plasma and of the cerebrospinal fluid, when the content of the protein in the plasma is reduced. There is also a similar decrease in the disparity of the sodium values.

## SUMMARY

1. When the plasma protein of dogs is lowered by plasmapheresis, the concentration of chloride and sodium in the plasma and the cerebrospinal fluid tends to become the same, a diminishing of the usual disparity which exists in the presence of a normal plasma protein.

2. This is in accord with the theory that the cerebrospinal fluid is a dialysate, with the choroid plexus acting as a simple dialyzing membrane.

We wish to thank Dr. Harvey Cushing and Dr. Otto Folin for their helpful suggestions, and for the opportunity of carrying on this research.

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## STUDIES ON TYPHUS FEVER\*

### IX. ON THE SERUM REACTIONS OF MEXICAN AND EUROPEAN TYPHUS RICKETTSIA

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(Received for publication, June 10, 1932)

In a communication published in April, 1932 (1), the writers described a method of obtaining large numbers of *Rickettsiae* by the inoculation of rats previously exposed to severe short wave-length

#### CORRECTION

In Vol. 56, No. 3, September 1, 1932, page 422, line 15 under Summary, for *After transfer to media suitable for acid growths*, read *After transfer to media suitable for acid-fast growths*.

Agglutination with typhus *Rickettsia* suspensions has been carried out by Otto and Dietrich, (3), by Weigl (4), by Krukowski (5), and by da Rocha-Lima (6). All of these observers use suspensions of the carbolized intestinal contents of infected lice (Weigl vaccine), a material containing much extraneous debris but rich in *Rickettsia* and, with proper precautions, quite suitable for agglutination experiments. All of them found that the blood of typhus convalescents developed agglutinins for such suspensions, Krukowski stating that the *Rickettsia* agglutinins appeared earlier and attained higher potencies than did the agglutinins for *Proteus* X-19. This investigator also observed that guinea pigs—animals on which the Weil-Felix reaction rarely appears—developed *Rickettsia* agglutinins. Reviewing these

\* The work here reported was carried out in part on a grant from the De Lamar Mobile Research Fund.

experiments, Otto and Munter (7) express the opinion that *Rickettsia* agglutination is more specifically diagnostic of typhus fever than is the Weil-Felix reaction.

All such experiments up to the present time have been done with the European variety of typhus and with *Rickettsiae* obtained from lice. The etiological rôle of these organisms, however, is not in any way questioned today. There are still investigators, on the other hand, who are not yet convinced of the specific significance of the organisms observed in the tunica cells of Mexican typhus guinea pigs (see Nicolle and Laigret cited above). It was for the purpose of elucidating this point and to investigate the serological relationships between the Mexican (New World) and Old World varieties of the disease that the following experiments were undertaken.

### *Technique*

The materials used for agglutination were the Weigl vaccine, of which we obtained several ampoules from Dr. Mooser, and our own suspensions of Mexican *Rickettsia* produced by the rat X-ray method referred to above.

In a large series of preliminary experiments we discovered that the formalin-treated *Rickettsiae*, as described in our published technique, were not as readily agglutinable as were similarly prepared carbolyzed suspensions. The reason for this is not clear, but in all the protocols cited the material used was carbolyzed (0.5 per cent phenol).

We devised a number of agglutination methods in which the organisms were observed microscopically and macroscopically, with and without staining; and while some of these were reasonably satisfactory and gave us much preliminary information, we report below only the results obtained by our final method, of the reliability of which we are satisfied. Microscopic methods were eventually given up entirely as possessing too many pitfalls of false clumping under conditions of prolonged observation. The method as finally adopted depends upon the use of small, flat tubes especially made for us, which can be used with minute quantities of material (this for reasons of economy) and which can be observed with convenience under a binocular of 30 diameters' magnification. Serum dilutions and *Rickettsia* suspensions are run into the tubes with capillary pipettes, and mixed by drawing them in and out several times. The tubes are then incubated in a water bath at 40°C. and observed at intervals of 2 to 3 hours. Agglutination was invariably slow, rarely clear—even with strong sera—in less than 2 hours. After 5 hours, agglutination was usually complete. At about that time the tubes were left at room temperature for subsequent reading and were again read after a night in the ice chest. Readings were independently made by two, and sometimes by three observers. All readings were made by comparing the tube under observation with a series of controls.

In the code used, + means slight agglutination, but definite; ++ means strong agglutination with some turbidity left between clumps; +++ means almost complete agglutination; ++++ means complete agglutination; - means negative agglutination; 0 means that this particular dilution was not set up.

### EXPERIMENTS

Our first experiments concerned themselves with agglutinin development in guinea pigs convalescent from infection with a European (Breinl) strain of typhus, a Tunisian strain, and our own Mexican strain.

*Experiment I.*—The occasional omission of a dilution (0) in the comparative series was due to the necessity of economy with the Weigl vaccine, of which we had only a meagre supply.

The sera listed in the tables below are as follows:

*Mexican Guinea Pig 1.*—Bled 29 days after inoculation, 15 day after defervescence, 17 days after subsidence of swelling.

*Mexican Guinea Pig 2.*—Bled 21 days after inoculation, 2 days after defervescence, 9 days after subsidence of swelling.

*European Guinea Pig 1.*—Bled 17 days after inoculation, 4 days after defervescence.

*European Guinea Pig 1a.*—Same animal bled 6 days later.

*European Guinea Pig 2.*—Bled 23 days after inoculation, 10 days after defervescence.

*European Guinea Pig 2a.*—Same guinea pig bled 8 days later. None of the sera employed were heated.

In Table I, which shows the results, the sera are set up against the Weigl vaccine in 1-40 dilution only, a potency indicated by preliminary experience, and the controls are selected to give adequate safeguard without waste of material.

The foregoing experiment confirmed preliminary ones in showing that the sera of guinea pigs convalescent from either the Mexican or European typhus infection agglutinated both varieties of *Rickettsiae*. It was noticeable here, as in the earlier experiments, that the Mexican serum usually agglutinated the Weigl vaccine more powerfully than the European serum agglutinated our own *Rickettsia*.

*Experiment II.*—For the foregoing reason, it seemed desirable to us to make a comparison between two of the above sera in greater detail, setting up reactions at relatively short dilution intervals. A cross-agglutination of Mexican *Rickettsia* and of Weigl vaccine with convalescent guinea pig sera was therefore carried out. The results of this experiment are shown in Table II.

TABLE I

Serum	Dilution	Mexican <i>Rickettsia</i>	Weigl vaccine
Mexican Guinea Pig 1	1-40	++++	++ to +++
	1-80	++++	0
Mexican Guinea Pig 2	1-40	++++	++ to +++
	1-80	++++	0
European Guinea Pig 1	1-10	++	0
	1-20	++	0
	1-40	+	+++ to ++++
	1-80	—	0
European Guinea Pig 1a	1-10	+++	0
	1-40	0	+++
	1-80	—	0
European Guinea Pig 2	1-10	+++	0
	1-40	0	+++
	1-80	—	0
European Guinea Pig 2a	1-10	+++	+++ to ++++
	1-40	++	+++
	1-80	—	—
Normal Guinea Pig 1	1-10	—	0
	1-20	—	0
	1-40	—	—
Normal Guinea Pig 2	1-10	—	0
	1-20	—	0
	1-40	—	—
Normal Guinea Pig 3	1-20	—	—
	1-40	—	—
	1-80	—	0
Normal Guinea Pig 4	1-20	—	—
	1-40	—	0
	1-80	—	0

It was again noticeable that the Weigl vaccine was more quickly and completely agglutinated by the Mexican serum than was our own suspension by the European serum. While this may be due to im-

munological conditions, it is not impossible that it is partly a consequence of physical circumstances, since the Weigl material consists of a coarsely granular and heavy suspension, whereas our own *Rickettsia* vaccine is a much more finely divided suspension, particles under the binocular being exceedingly small.

TABLE II

Serum	Dilution	Mexican <i>Rickettsia</i>	Weigl vaccine
Mexican Guinea Pig 1	1-6	+++	+++
	1-10	++++	++++
	1-20	+++	++++
	1-30	+++	+++
	1-40	++++	++
	1-50	++++	+
	1-80	+++	+
	1-100	+++	
			+++ to +++++
European Guinea Pig 2	1-6	+	++++
	1-10	++	++++
	1-20	+++	++++
	1-30	++	++++
	1-40	++	++++
	1-50	++	+++
	1-80	-	+++
	1-100	-	
			-
Normal Guinea Pig 5	1-6	-	-
	1-20	-	-
	1-40	-	-
Normal Guinea Pig 6	1-6	-	-
	1-20	-	-
	1-40	-	-
Salt solution control		-	-

These and other reactions with guinea pig sera were sufficiently sharp to convince us that guinea pig convalescent serum from both varieties of typhus had acquired agglutinating properties for both types of *Rickettsiae*.

Under ordinary circumstances these experiments would convince us that our *Rickettsiae* are truly concerned with typhus and that vac-



cination against the disease with these suspensions is a rational procedure. The observations of Nicolle and Laigret, however, have suggested the possibility that the tunica organisms studied by Mooser and by ourselves might be adventitious saprophytes present in the stock guinea pigs and rats, stimulated into multiplication by the insult of intraperitoneal injections. In that case our agglutination experiments with guinea pig sera would carry little conviction.

We proceeded, therefore, to study human convalescent sera in the same manner in which we had studied the guinea pig sera. Our materials were as follows:

1. A Polish convalescent serum obtained through Dr. Mooser (Weil-Felix = 1-1280).
2. A Tunisian convalescent protective serum (Weil-Felix = 1-1280).
3. A Mexican convalescent serum, A, sent us by Dr. Mooser (Weil-Felix = ++ 1-3200).
4. A Mexican convalescent serum, B (Weil-Felix = 1-500, weakly 1-1000).
5. Two Mexican sera which were very old—over 2 years—and gave weak Weil-Felix reactions. Mexican Serum 7 (Weil-Felix = +++ 1-80). Mexican Serum 9 (Weil-Felix = +++ 1-160).

*Experiment III.*—Table III shows the results of agglutination experiments of human convalescent sera tested against our own carbolized *Rickettsia* suspension.

This experiment shows definite agglutination of our *Rickettsia* by all the convalescent sera, whatever their origins. But it is noticeable that the European sera agglutinate the Mexican organisms much more feebly than do the homologous sera, and there is apparent, in the European sera, a prozone of exceptional breadth. This may be due to the age of these sera. It will be remembered that Shibley (8) has shown that heating agglutinating sera is apt to extend the prozone considerably, and it is reasonable to assume that ageing may do the same thing. Nevertheless, we decided to run these sera out to their agglutinating limits and to so control them that we might make sure that the positive reactions in high serum dilutions were not non-specific phenomena.

*Experiment IV.*—Table IV represents consolidated titrations of two consecutive experiments done with identical materials.

Here again the zones are apparent in the Old World sera much more than in the Mexican, but there could be not the slightest question

TABLE III

Serum	Dilution	Result
Polish convalescent serum	1-20 1-80 1-160	$\pm$ + to ++ ++ to +++
Tunisian convalescent serum	1-20 1-80 1-160	+ ++ +
Mexican convalescent Serum A	1-20 1-80 1-160	++++ ++ ++
Mexican convalescent Serum B	1-20 1-80 1-160	+ ++++ ++ to +++
Mexican convalescent Serum 7	1-20 1-80	+ to ++ +
Mexican convalescent Serum 9	1-20 1-80	$\pm$ +
Normal human Serum 1	1-20	-
Normal human Serum 2	1-20	-

TABLE IV

Dilution	Sera								Normal 7 1-10-	Normal 8 1-10-
	Polish	Tunisian	Mexican A	Mexican B	Normal 3	Normal 4	Normal 5	Normal 6		
1-20	-	-	++++	++	-	-	-	-	-	-
1-50	-	-	+++	+	-	-	-	-	-	-
1-100	$\pm$	-	+++	+	-	0	0	0	0	0
1-200	$\pm$	$\pm$	+	++++	0	0	0	0	0	0
1-300	++	$\pm$	$\pm$	++	0	0	0	0	0	0
1-400	+	+	+	+++	0	0	0	0	0	0
1-500	++	++	+	0	0	0	0	0	0	0
1-750	+++	+ to ++	0	0	0	0	0	0	0	0
1-1000	+	+	0	0	0	0	0	0	0	0

about the ++ and +++ agglutinations of the Mexican *Rickettsia* which occurred in the Old World serum dilutions above 1-200.

TABLE V

Human serum	Dilutions	Mexican <i>Rickettsia</i>	Weigl vaccine
Mexican Serum D	1-20	—	—
	1-50	—	—
	1-100	—	—
Mexican Serum E	1-20	—	—
	1-50	—	—
	1-100	—	±
Mexican Serum F	1-20	+++	+++
	1-50	+++	+++
	1-100	+++	+++
	1-200	+ to ++	0
	1-500	++	0
	1-1000	+	0
Mexican Serum G	1-10	++++	++++
	1-20	++++	++++
	1-50	+++	+++
	1-100	+++	++++
Normal Serum 5	1-10	—	—
	1-20	—	—
	1-50	—	—
	1-100	—	—
Normal Serum 7	1-10	—	0
	1-50	—	0
Normal Serum 8	1-100	±	0
	1-200	—	0
	1-500	—	0
	1-1000	—	0

*Experiment V.*—At this time, May, 1932, we made an observation which convinced us that agglutination of our *Rickettsia* in human serum indicated typhus. Dr. Varela of the Mexican Institute of Hygiene was good enough to send us four convalescent sera supposed to have been taken from patients at the period of defervescence.

Agglutinations done with these sera and with both varieties of *Rickettsia* vac-

cines are shown in Table V, which is a consolidated tabulation of two experiments carried out on consecutive days.

The results of this experiment were disquieting, since all the sera received were supposed to have come from hospital cases diagnosed as typhus fever, or tabardillo. Weil-Felix reactions were therefore promptly carried out with these specimens. The results are shown in Table VI.

The correspondence of the Weil-Felix reactions of these sera with their agglutination power for both the Mexican *Rickettsia* and the European louse *Rickettsia* is so striking that we have no hesitation in concluding that we are dealing with specific immunological phenomena.

Although the experiments described in the preceding paragraphs appeared to us to eliminate any possible uncertainty as to the etiological relationship of the Mexican tunica *Rickettsia* to the disease in human beings, there was obviously another line of simple experimentation by which this important fact could be further consolidated and by which the relationship between the two types of agent—the Old World *Rickettsia* of the louse (as found in the Weigl vaccine) and our own *Rickettsia*—could be investigated. This consisted in immunizing rabbits with each of the two vaccines respectively and carrying out cross-agglutinations, a procedure made possible by the fact that we were still in possession of a few phials of the carbolized louse vaccine prepared by Weigl. Accordingly, the following experiment was carried out.

*Experiment VI.*—Two rabbits were injected as follows:

*Rabbit 1.*—Weil-Felix negative. Was intravenously injected three times, at 5, 6, and 7 day intervals, with 3 cc. of our formalized X-ray rat vaccine of Mexican *Rickettsia*. 11 days after the last injection the serum of this rabbit gave a Weil-Felix: 1-20 + + + +; 1-40 + + +; 1-80 +.<sup>1</sup>

*Rabbit 2.*—Weil-Felix negative. Was intravenously injected three times, at 5 day intervals, with 1 cc. of a Weigl European louse *Rickettsia* vaccine. A small specimen of blood was taken from the ear of this animal 5 days after the last injection. Although this was obviously too early for the most potent results, the

<sup>1</sup> Tested on guinea pigs this serum gave no protection against the Mexican disease, a fact in contrast with similarly prepared horse serum to be discussed in another paper but in harmony with similar rabbit serum observations made by Brienl.

serum gave a Weil-Felix: 1-20 +++; 1-40 ++; 1-80 +. For this reason we decided to use it for the experiment here recorded.

We set up a complete set of cross-agglutinations with the two sera and their respective vaccines. As will be seen, each serum highly agglutinated its homo-

TABLE VI  
*Weil-Felix Reaction (Proteus X-19)*

Dilution	Mexican Serum D	Mexican Serum E	Mexican Serum F	Mexican Serum G
1-20	—	—	++++	++++
1-40	—	—	++++	++++
1-80	—	—	++++	++++
1-160	—	—	++++	++++
1-320	—	—	++++	++++
1-640	—	—	++++	++++

TABLE VII

*Agglutination of Mexican Rickettsia and Weigl Vaccine (European Louse Rickettsia) by Rabbit 1 Immunized with Mexican Vaccine from X-Rayed Rats*

Serum Rabbit 1 before immunization (Weil-Felix negative)	Mexican <i>Rickettsia</i>	Weigl vaccine
1-10	—	—
1-50	—	—
1-100	—	—
1-200	—	—
1-500	—	—
1-1000	—	—
Serum Rabbit 1 after immunization (Weil-Felix 1-40)	Mexican <i>Rickettsia</i>	Weigl vaccine
1-10	+++	++++
1-50	+++	++
1-100	+++	+++
1-200	+++	+
1-500	+++	—
1-1000	++	—

gous *Rickettsia*, but since, in each case, the rabbit had received, with the vaccines, a certain amount of either rat or louse protein, these results might justly be criticized. Agglutinations of either one of the vaccines by the heterologous serum cannot be subject to this objection. The reactions are shown in Tables VII and VIII.

Tables VII and VIII illustrate that the serum of a rabbit immunized with our formalinized Mexican *Rickettsia* vaccine will agglutinate the Weigl louse *Rickettsia* and that the serum of a rabbit immunized with the European louse vaccine develops agglutinating power for the Mexican *Rickettsia* as obtained by us from guinea pigs through X-rayed rats. Incidentally, the appearance of these agglutinins coincided in both animals with the appearance of Weil-Felix reactions.

TABLE VIII

*Agglutination of Mexican Rickettsia and of Weigl Vaccine by Serum of Rabbit 2 Immunized with European Louse Vaccine (Weigl)*

Serum Rabbit 2 before immunization (Weil-Felix negative)	Mexican <i>Rickettsia</i>	Weigl vaccine
1-10	—	+
1-50	—	—
1-100	—	—
1-200	—	—
1-500	—	—
1-1000	—	—
Serum Rabbit 2 after immunization (Weil-Felix 1-40)	Mexican <i>Rickettsia</i>	Weigl vaccine
1-10	+++	++++
1-50	+++	++++
1-100	+++	++++
1-200	+++	++++
1-500	++	++++
1-1000	+	+++

## DISCUSSION

The experiments recorded above seem to permit little further doubt that the *Rickettsia* suspensions obtained by us in prepared rats inoculated with tunica material from Mexican typhus guinea pigs represent organisms specifically related to the Old World typhus fever in man and closely related to, if not identical with the *Rickettsia prowazeki* seen in lice that have fed on cases of Old World typhus. Even though objections might be raised to this conclusion on the basis of our guinea pig serum agglutinations, it is not likely that an organism saprophytic

in guinea pigs and rats should also be present side by side with an undiscovered typhus virus in human beings infected with both the New World and the Old World diseases. Further than this, the agglutinations carried out with the sera of rabbits immunized with the two types of *Rickettsia* seem to us conclusive evidence that the European louse *Rickettsia* and the Mexican tunica organism are either identical or closely related. There is no other reasonable interpretation of these cross-agglutinations, and we can see no source of error in the experiments. If these results are considered together with the facts that, in both cases, the ultimate sources of the organisms are animals infected with material from active human cases, and that the rabbits developed the Weil-Felix reaction, which is acknowledgedly diagnostic of the human typhus group, the chain of evidence linking these organisms with the diseases seems complete.

Whether the comparative agglutinations indicate an antigenic overlapping of the *Rickettsiae* of the two sources we cannot decide with certainty on the basis of these tests, since the two antigens at our disposal—our rat vaccines and the Weigl louse vaccine—are quite incomparable in gross physical properties. However, from the differences in agglutinating titre manifested by individual European strain and Mexican strain guinea pig sera respectively upon one and the same vaccine, we are inclined to believe that there is a definite, though slight and overlapping, antigenic difference which must be taken into consideration in connection with the practical problems of specific prophylaxis and serum production.

#### CONCLUSIONS

1. The blood of guinea pigs convalescent from Old World and New World typhus infection develops agglutinating properties for the tunica and rat *Rickettsiae* of the New World diseases and for the louse *Rickettsia* of the Old World disease.
2. The two microorganisms are closely related, though probably not identical.
3. Human convalescents of both varieties of typhus develop agglutinins for both types of *Rickettsiae*. Such *Rickettsia*-agglutinating properties are parallel with the Weil-Felix reaction in the human sera.
4. Rabbits immunized with Weigl louse vaccines develop agglu-

tinins for our X-ray rat vaccines and *vice versa*. In both cases the rabbit sera develop agglutinins for *Proteus* X-19.

5. These experiments furnish a further and, we believe, conclusive proof of the etiological rôle, in New World typhus fever, of the *Rickettsia* bodies first seen in the tunica cells of inoculated guinea pigs by Mooser, and obtained in massive amounts by ourselves.

6. The serum reactions also provide a further logical basis for experiments in prophylactic vaccination with these *Rickettsiae*.

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# THE IMMUNOLOGICAL BEHAVIOUR OF THE SECOND PROTEIN (LIVETIN) OF HEN'S EGG YOLK

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Protein constitutes an essential and quantitatively large part of the food provided by the maternal organism both for the nutrition of the very young mammal and that of the avian embryo. It is therefore not unnatural that the chemical and immunological relationships existing between the proteins of the maternal tissues and those rapidly secreted, in the milk or the egg yolk or white, for the nutrition of the offspring, should have received attention. With regard to milk, the immunological similarities between the proteins of this fluid and of bovine serum were reviewed in 1921 by Wells and Osborne (1). To summarize the present situation very briefly, it has been shown that the caseins of different animals are closely related though not identical biologically and chemically, while the whey proteins are species-specific. The latter comprise three distinct antigens—lactalbumin, lactoglobulin and an alcohol-soluble protein. Lactoglobulin alone of the four milk proteins will produce positive biological reactions against bovine blood serum. Anaphylactic, precipitin and complement fixation tests have been used by many workers in this field with fairly concordant results. It may be taken as almost certain that milk globulin and serum globulin are identical proteins (Sasaki (2)), having a common origin *outside* the mammary gland, whilst milk albumin and casein are built up *in* the gland either from simple amino acids or from blood proteins which are altered out of all recognition in the process.

With regard to the egg proteins, it has long been known that egg white contains an antigen common to it and to fowl serum (Uhlenhuth (3), Levene (4), Gengou (5)). It remained however, for Hektoen and Cole (6) to show that, while egg white contained five distinct antigens (ovoglobulin, ovomucin, crystalline ovalbumin, non-crystalline conalbumin and ovomucoid), only the conalbumin was immunologically identical with any of the fowl's serum proteins; namely, with the serum albumin.

The proteins of the hen's egg yolk have not been so closely investigated. Wells (7) showed that ovovitellin was an antigen distinguishable by anaphylaxis from ovomucoid and ovalbumin, while Emmerich (8) found by the precipitin test that fish and turtle egg yolk proteins would give positive reactions against anti-hen yolk serum. Seng (9) found that the serum of rabbits immunized against hen's egg yolk gave precipitin reactions with saline suspensions of the yolks of the eggs of

many species of birds. The distribution of positive reactions, however, was very wide, and did not show family differences or gradations, suggesting, perhaps, that yolk contains common (similar to casein) and specific (similar to whey protein) antigens.

There are two distinctly different proteins in hen's egg yolk. One, ovovitellin, a phosphoprotein, is physically quite unlike any of the constituent proteins of the serum. The other, livetin, possesses physical properties which strongly recall those of serum pseudoglobulin (Kay and Marshall (10)). Since the eggs of both vertebrate and invertebrate animals are characterized by a yolk, and since in all the three species of animal so far examined (10, 29) a pseudoglobulin is present in the egg yolk, it appeared to us that it might be of some significance, possibly in the inheritance of immunity, if this second protein of egg yolk were identical with one of the globulins of the maternal serum.

The three main proteins of serum have been shown to be distinct antigens by several workers, most clearly by Hektoen and Welker (11), who used the precipitin test and employed beef, dog, horse and human serums. Chemical evidences of difference are less clear. It is generally admitted that serum albumin is chemically as well as physically distinct from serum globulin. However, chemical differentiation between serum euglobulin and pseudoglobulin is still a matter of dispute, and many methods have been used in an effort to distinguish between them. Obermayer and Willheim (12) used the ratio of the formol titration value to the total nitrogen; Grøh and Faltin (13) used absorption spectography; electrodialysis and the anaphylactic test were employed by Otto and Iwanoff (14); von Mutzenbecher (15) used electrodialysis and the ultracentrifuge, and Hartley (16) employed the method of Van Slyke. Nevertheless, the observations of Chick (17), who suggested that serum euglobulin is a protein-lipoid complex of a pseudoglobulin solution with a lipid emulsion, are re-echoed in the review by Howe (18) and in Wells's text-book (19), and are reiterated by Went and Farago (20). Sørensen (21) found euglobulin not changed in solubility by extraction with alcohol-ether. Hewitt (22) reported that lipin-free pseudoglobulin and euglobulin have distinctly different optical rotatory powers, and hence euglobulin cannot be a mechanical complex of pseudoglobulin and lipin. Finally Svedberg and Sjögren (23) have stated, on the basis of their work with the ultracentrifuge, that serum globulin is homogeneous with regard to molecular weight, and that euglobulin and pseudoglobulin are "laboratory products."

The opinion of Chick (17) that euglobulin in serum may be a complex of pseudoglobulin and lipoids, coupled with the well known fact that lipins may modify the antigenic specificity of proteins (Wells (7)) led us, in our later experiments, to use Hewitt's (22) modification of the methods of Hardy and Gardiner (24), Young (25)

and Hartley (26), for the removal of bound lipins from proteins at low temperatures, thus avoiding denaturation, for the preparation not only of livetin but of the serum globulins.

In contrast to Hewitt, we found that the distinctive property of euglobulin of precipitation by ordinary dialysis disappeared after cold extraction. However, it was still possible to separate serum globulin into two fractions by 33 and 50 per cent saturation with ammonium sulfate.

Our experimental findings may be grouped under four heads: (1) anaphylactic (first series), (2) precipitin, (3) complement fixation, (4) anaphylactic (second series).

### 1. Anaphylactic Tests (First Series)

(a) *Preparation of Proteins. Livetin.*—Egg yolks were carefully washed in 0.9 per cent sodium chloride and rolled over fine linen to separate all adherent white. The vitelline membrane was then ruptured, and its contents allowed to flow out. The membrane was then discarded, and the yolk mixed with an equal volume of 8 per cent NaCl solution and shaken with repeated changes of ether to dissolve out free lipoids. The residual solution contained the proteins lecithovitellin and livetin. It was dialyzed against tap water, when the lecithovitellin precipitated, the livetin remaining in solution. The livetin solution was then centrifuged off, clarified by filtration through paper-pulp and further purified by repeating thrice the operations of precipitating by half saturation with ammonium sulfate, centrifuging, redissolving by adding water, and filtering. After this, the lipins were removed by alcohol-ether extraction at  $-15^{\circ}$  (19), the protein redissolved in water, filtered and reprecipitated, dialyzed against distilled water until  $\text{SO}_4$ -free, and preserved by adding either an equal volume of glycerol, or one one-hundredth of the volume of 1 per cent sodium merthiolate (27).

*Serum Proteins.*—Chicken serum was half saturated with ammonium sulfate, and the precipitated proteins were washed with half-saturated ammonium sulfate. The globulins were then separated from each other by dialysis against distilled water, the euglobulin precipitating out. They were ether-alcohol-extracted at  $-15^{\circ}$ . The albumin was precipitated by saturating the original globulin filtrate with ammonium sulfate.

(b) *First Experiment.*—A group of young guinea pigs was sensitized with intraperitoneal injections of livetin, using from 0.1 to 0.4 mg. of the protein, and another group with hen serum, using from 0.2 to 0.4 cc. From 24 to 29 days later, shocking injections were made into the internal jugular vein. The results may be briefly stated as follows:

6 guinea pigs sensitized with livetin:

2 were reinjected with livetin 24 days later, one dying from typical anaphylaxis, the other being unaffected.

4 were injected with serum 24 days later, 3 of these dying in anaphylactic shock, the other recovering after severe dyspnea.

10 guinea pigs sensitized with serum:

1 was injected 24 days later with serum and died from anaphylaxis.

1 was injected 28 days later with livetin and died from anaphylaxis.

7 were injected 28 to 35 days later with livetin, and exhibited varying degrees of shock from which they recovered. 3 of these died from anaphylaxis upon injection of serum within an hour after injection of livetin.

All guinea pigs dying in this and subsequent experiments from apparent anaphylaxis were autopsied immediately after death to reveal the maximally distended lungs typical of this species in anaphylactic shock.

(c) *Second Experiment.*—In this experiment the individual serum proteins were used, the sensitizing proteins other than livetin being prepared from hen serum and shocking proteins from rooster serum.

*Results.*—

8 guinea pigs sensitized with livetin:

Killed with pseudoglobulin. . . . . 2

Killed with serum albumin. . . . . 2

Killed with serum euglobulin. . . . . 1

Acute shock with pseudoglobulin. . . . . 1

No shock with serum albumin or livetin. . . . . 1

No shock with euglobulin, slight shock from livetin. . . . . 1

2 sensitized with serum albumin:

Killed with livetin. . . . . 1

No shock with livetin, slight shock with serum albumin. . . . . 1

4 sensitized with serum euglobulin:

Killed with livetin. . . . . 3

Killed with serum albumin. . . . . 1

2 sensitized with serum pseudoglobulin:

Killed with livetin. . . . . 1

Killed with serum albumin. . . . . 1

The above findings, while giving strong indications of a relationship between the proteins used, appeared to be obscured either by (1) poor separation of the serum proteins from each other, or (2) too great sensitivity of the test to traces of contaminants. Accordingly the precipitin test was next employed (further anaphylactic experiments are mentioned later).

## 2. Precipitin Tests

(a) *Preparation of Proteins.* *Livetin.*—As previously described.

*Serum Proteins.*—Several liters of blood were obtained from a wholesale killing of cockerels at a local packing house. The serum was half-saturated with ammo-

nium sulfate, filtered and the filtrate completely saturated with  $(\text{NH}_4)_2\text{SO}_4$ . The precipitate was redissolved in water and the  $(\text{NH}_4)_2\text{SO}_4$  concentration brought up to 50 per cent, the solution then filtered and fully saturated. This was repeated five times, the final albumin solution allowed to stand for 2 weeks, clarified and dialyzed against distilled water. The precipitated mixed globulins were reprecipitated five times, extracted with alcohol-ether at  $-15^\circ$ , the euglobulin separated by precipitating with 33 per cent  $(\text{NH}_4)_2\text{SO}_4$ , repeating the precipitation at this level five times, dialyzing the resultant euglobulin against distilled water and raising its saline concentration to 0.9 per cent. The pseudoglobulin was prepared by clarifying the centrifugate from the first 33 per cent precipitation, raising the concentration to 50 per cent and centrifuging off the precipitated pseudoglobulin, redissolving in water and repeating the process until the solution remained absolutely clear on raising its saturation to 33 per cent. The pseudoglobulin was then dialyzed for 10 days against repeated changes of distilled water and clarified by centrifuging.

(b) *Technique*.—Precipitin tests were made by adding the antigen in increasing dilutions (physiological saline) to the undiluted serum, the latter being layered under the antigen. After  $\frac{1}{2}$  hour at room temperature, or 15 minutes at  $37^\circ$ , the tubes were read, usually by daylight.

(c) *First Experiment*.—A rabbit was immunized to livetin by means of five injections of 2 cc. of 0.5 per cent livetin solution at 3 day intervals. After a 3 week interval the animal was bled and the serum preserved with 0.01 per cent sodium merthiolate. (This serum was still clear and potent 5 months later.) A positive reaction was obtained up to a dilution of 1:600,000 to 1:800,000 of livetin, 1:100,000 of serum albumin, 1:100,000 of euglobulin and 1:800,000 of pseudoglobulin (all in physiological saline solution).

(d) *Second Experiment*.—Three rabbits were immunized to hen serum by receiving three injections of 5 cc. of hen serum in 50 per cent glycerol at 4 day intervals. 18 days later they were bled. The serum thus obtained gave positive tests with dilutions of hen serum down to 1:10,000, negative with 1:100,000, positive with livetin down to 1:10,000 and negative with 1:100,000.

(e) *Third Experiment*.—The albumin was roughly separated from the globulin of a quantity of fresh serum, obtained as in (a), diluted three times with physiological saline, by 50 per cent saturation with ammonium sulfate. The mixed globulins were dialyzed and then alcohol-ether-extracted. The three proteins—albumin, pseudoglobulin and euglobulin—were then purified by repeated  $(\text{NH}_4)_2\text{SO}_4$  precipitation according to the method of Hektoen and Welker (11), the albumin fraction being taken as that precipitating between 66 and 100 per cent saturation.

#### Results.—

Rabbit vs. hen serum and	In dilution of			
	1:10,000	1:20,000	1:30,000	1:40,000
Pseudoglobulin.....	+	+	+	±
Livetin.....	+	+	±	—

Rabbit vs. livetin serum and	In dilution of							
	1:1,000	1:4,000	1:10,000	1:50,000	1:100,000	1:200,000	1:400,000	1:600,000
Euglobulin.....	+		+		+	+	+	±
Albumin.....		+	+	+	±	—		

(f) *Fourth Experiment.*—An immune serum was prepared for each of pseudoglobulin, euglobulin and whole fresh egg white by injecting at 4 day intervals for 3 weeks into rabbits the following quantities of the antigens:  $1\frac{1}{2}$  to 2 cc. of 0.2 per cent pseudoglobulin, 2 to 5 cc. of 0.32 per cent euglobulin, and  $1\frac{1}{2}$  to 2 cc. of ordinary egg white in 50 per cent glycerol. Two rabbits were used for each protein. 10 days after the last injection the animals were bled, and in each case the serum reacted strongly with the immunizing protein. Egg white was included merely for the purpose of comparing the behaviour of an unpurified, unextracted protein mixture with that of the purified antigens.

*Results of Titration.*—

(1) Rabbit vs. pseudoglobulin serum and

Pseudoglobulin	Livetin	Euglobulin	Egg white	Serum albumin
1:5,000 ++	1:5,000 ++		1:10 +	1:4,000 —
1:10,000 ++	1:10,000 ++	1:10,000 ++	1:100 —	1:10,000 —
1:100,000 +	1:100,000 ±	1:100,000 +	1:1,000 —	1:50,000 —
1:500,000 —		1:500,000 —		1:100,000 —

(2) Rabbit vs. egg white serum and

Egg white	Livetin	Pseudoglobulin	Euglobulin	Serum albumin
1:1,000 +++++	1:1,000 —	1:5,000 —	1:5,000 —	1:4,000 ++
1:10,000 +++++	1:5,000 —	1:10,000 —	1:10,000 —	
1:100,000 +	1:10,000 —			
1:500,000 —				
1:1,000,000 —				

(3) Rabbit vs. euglobulin serum and

Euglobulin	Livetin	Pseudoglobulin	Egg white
1:10,000 +++++	1:5,000 ++	1:10,000 ++	1:10 +
1:100,000 ++	1:10,000 +	1:100,000 +	1:100 +
1:500,000 —	1:100,000 —	1:500,000 —	1:1,000 —

(4) Rabbit vs. livetin serum (from (c) above, now 14 wks. old) and

Livetin	Egg white		Euglobulin		Pseudoglobulin		Serum albumin
1:10,000	++++	1:10	+	1:10,000	+	1:100,000	+
1:100,000	++	1:100	±	1:100,000	+	1:200,000	±
1:200,000	+	1:1,000	+	1:200,000	+	1:300,000	—
1:300,000	±	1:10,000	+	1:300,000	—		
1:500,000	—	1:100,000	—				
		1:500,000	—				

*Results.*—All the above tests were controlled by (1) antigens against normal serum, (2) immune serum against saline. The results indicate close relationship between livetin and the two serum globulins, a less close relationship between egg white and the serum globulins, a possible relationship between livetin and some protein or proteins in egg white, and no differentiation between the two serum globulins by means of the precipitin test.

Accordingly, the complement fixation test was next employed, as probably the most quantitative and delicate test for establishment of protein identity that we have at our disposal, though it is no less purely empirical than the two methods previously used.

### 3. Complement Fixation Tests

The facilities of the serology laboratories of the Department of Pathology were placed at our disposal by Dr. H. K. Detweiler and Miss E. Paul for the carrying out of these tests. We gratefully acknowledge this privilege.

(a) *Antigens.*—The same serum proteins were used as in paragraph 2 (c) above. The livetin was the same as used throughout, while the whites of fresh eggs, preserved in 0.02 per cent sodium merthiolate, were used when egg white is specified. For convenience of comparison, 0.01 mg. of protein is referred to as a unit of antigen.

*Antisera.*—The same antisera were used as in the precipitin experiments. The following precautions were taken. (1) All antigens and antisera were heated for  $\frac{1}{2}$  hour at 56° to destroy complement; (2) volumes were always brought up to 2 cc. with normal saline before addition of complement, 3 units of which were always used; (3) antigens were titrated in absence of serum, and not more than one-quarter of the maximum non-anticomplementary dose as thus determined were used in actual tests; (4) sera were always tested for anticomplementary properties; (5) diluted antisera were titrated in increasing doses against their antigen (in excess)



TABLE I  
Comparison of Livetin with Fowl Serum Proteins by the Complement Fixation Test  
Experiment 4

No. of tubes	No. of units of antigen	Antigen	Serum	Result	Remarks
6 1	20-120 140	Pseudoglobulin "	None "	0* 1	
6 1	32-224 288	Livetin "	" "	0 4	
8	10-100	Euglobulin	"	0	
4	5-25	Serum albumin	"	0	
5 8	10	None Euglobulin	0.1 to 0.8 cc. rabbit vs. euglobulin 0.1 " 1.0 " " "	0 4	Use 0.3 cc. serum in tests
4		None	0.1 " 0.5 " " " pseudoglobulin	0	Absence of anticomplementary power
1 1 1 1 1 1 1	20 20 20 20 20 20 20	Pseudoglobulin " " " " "	0.7 cc. 1 in 10 " " 0.6 " 1 " 10 " " 0.5 " 1 " 10 " " 0.4 " 1 " 10 " " 0.3 " 1 " 10 " " 0.2 " 1 " 10 " "	4 2 2 1/0 0 0	Use 1.0 cc. 1:10 in tests
1 1 1 1 1	32 48 64 96 128	Livetin " " " "	0.3 " rabbit vs. euglobulin 0.3 " " " 0.3 " " " 0.3 " " " 0.3 " " "	1 1 1/0 0 1	Livetin does not fix complement with anti-euglobulin and antipseudoglobulin serum. (The slight indications with anti-euglobulin serum cannot be taken as positive)

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TABLE II  
*Further Comparison of Livetin with Foal Serum Globulins by the Complement Fixation Test*  
*Experiment 5*

The possible relationship between euglobulin and pseudoglobulin was further explored in the following series.

No. of tubes	No. of units of antigen	Antigen	Serum	Result	Remarks
8	40-180	Pseudoglobulin	None	0	
3	160-288	Livetin	"	0	
1	320	"	"	1	
10	50-180	Euglobulin	"	0	
5	—	None	0.2 to 0.6 cc. rabbit vs. pseudoglobulin	0	
1	10	Pseudoglobulin	0.2 cc. rabbit vs. pseudoglobulin	0	
1	15	"	0.2 " " "	2	
1	20	"	0.2 " " "	3	
4	25-40	"	0.2 " " "	4	
1	10	Euglobulin	0.2 " " "	0	No fixation by euglobulin in the same concentrations that cause fixation in the case of pseudoglobulin
1	15	"	0.2 " " "	0	
1	20	"	0.2 " " "	0	
1	25	"	0.2 " " "	0	
1	30	"	0.2 " " "	0	
1	35	"	0.2 " " "	1/0	
1	40	"	0.2 " " "	0	
3	160-224	Livetin	0.2 " " "	0	

to determine the minimum fixative amount of serum; this amount was doubled for titration of protein.

(b) *Results*.—Experiment 1, summary of 60 tests: Rabbit *versus* livetin serum gave complete fixation with 1 to 10 units of livetin, no fixation with 1 to 10 units of serum pseudoglobulin, serum euglobulin or serum albumin.

Experiment 2, summary of 96 tests: Rabbit *versus* livetin serum gave no fixation with 16 to 100 units of serum euglobulin, no fixation with 20 to 80 units of serum pseudoglobulin, no fixation with 0.1 to 0.4 cc. of 1:10 hen serum (equivalent to 80 to 320 units of serum protein).

Experiment 3, summary of 174 tests: Rabbit *versus* egg white serum gave complete fixation with 2 to 25 units of egg white, no fixation with serum pseudoglobulin ranging from 5 units to 40 units, no fixation with serum euglobulin ranging from 5 units to 62 units, no fixation with livetin ranging from 8 units to 160 units.

Rabbit *versus* euglobulin serum gave no fixation with egg white ranging from 50 to 200 units, no fixation with 80 units of livetin.

Rabbit *versus* hen-serum serum was anticomplementary. (Was this an actual immunity against complement?)

Rabbit *versus* livetin serum gave no fixation with 50 to 300 units of egg white.

Experiments 4 and 5 are given in more detail in Tables I and II.

(c) *Conclusions from the Complement Fixation Test*.—When used as immunizing proteins, pseudoglobulin behaved as an antigen distinct from euglobulin, livetin, egg white or serum albumin; egg white behaved as an antigen distinct from pseudoglobulin, euglobulin and livetin; livetin behaved as an antigen distinct from egg white, pseudoglobulin or serum albumin; while euglobulin yielded a serum which was less sharply specific than the others, but was far more highly reactive for euglobulin than for livetin and pseudoglobulin, and did not react with egg white and serum albumin.

#### 4. Anaphylactic Tests (Second Series)

In these tests the same antigens were used as in the complement fixation tests. The sensitizing dose was 0.1 mg., administered intraperitoneally, and the shocking dose 1.0 mg., administered intravenously, of protein. Nine guinea pigs were sensitized with pseudoglobulin, and nine with euglobulin.

Animals sensitized with euglobulin:

Killed with livetin. . . . .	1
Severe shock with livetin. . . . .	1
Killed with pseudoglobulin. . . . .	1
Unaffected by serum albumin, killed by injecting euglobulin 30 min. later. . . . .	2

Unaffected by serum albumin, shocked severely 30 min. later by euglobulin.....	1
No sensitization.....	2
Killed with euglobulin.....	1
Animals sensitized with pseudoglobulin:	
Killed with livetin.....	3
Killed with euglobulin.....	1
No shock with serum albumin, killed 30 min. later with pseudoglobulin.	2
No sensitization .....	2
Killed with pseudoglobulin.....	1

These results reveal clearly (1) a relationship between livetin and serum globulin, (2) effective separation of albumin from the globulins of serum. Precipitin tests were repeated on the same day using the serum albumin preparation against rabbit *versus* livetin serum, and found to be strongly positive.

#### DISCUSSION

So many complex and unknown factors enter into the antibody reactions that a positive result is probably more significant than a negative one.<sup>1</sup> Hence we are inclined to look upon the positive evidence of the identity of livetin of the egg yolk and globulin of fowl serum afforded by both the anaphylactic and precipitin tests as probably outweighing the negative results of the complement fixation reactions.

The results in 2 (f), 3 (b) and 4, using the same antigen solution, provide interesting opportunities for the comparison of the behaviour of the three tests. Thus the euglobulin and pseudoglobulin of fowl serum were clearly differentiated by the complement fixation test, while both the anaphylactic and precipitin reactions did not distinguish between them. Again whilst serum albumin would not shock globulin-sensitized guinea pigs (in 4) which were sensitive to livetin, it would nevertheless give a precipitin reaction with the serum of rabbits which had been immunized against livetin.

Such discrepancies might provide the opponents of the unitarian hypothesis with an argument in favour of the existence of essential differences in the mechanism underlying the anaphylactic and the

<sup>1</sup> Cf. Wells (19), p. 112.

precipitin reactions on the one hand and the complement fixation test on the other. These discrepancies may, however, indicate that the former two types of reaction are not tests of absolute chemical and structural identity, but only of the identity of some important portion of the molecule of each protein (Wormall (28)), whereas a positive result from the last test (complement fixation) requires a still closer approximation to complete identity between the two proteins concerned. Knowledge of the relation of structure to specificity has been reviewed by Wells (19).<sup>2</sup> Without venturing further into speculation we shall conclude by drawing attention to the positive evidence of a close relationship which our results with the anaphylactic and precipitin tests would appear to indicate as existing between livetin and serum globulin.

#### SUMMARY

A comparison of the livetin of egg yolk and serum globulin of the common fowl using (a) anaphylactic and (b) precipitin reactions, indicates that the two proteins are very closely related, if not identical.

The complement fixation test does not give the same result but indicates a difference between the two proteins. The complement fixation test also shows differences between serum euglobulin and serum pseudoglobulin when these proteins, prepared with considerable care, cannot be distinguished either by anaphylactic or precipitin tests.

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# PROPERTIES OF THE CAUSATIVE AGENT OF A CHICKEN TUMOR

## VI. ACTION OF THE ASSOCIATED INHIBITOR ON MOUSE TUMORS\*

By JAMES B. MURPHY, M.D., AND ERNEST STURM

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(Received for publication, June 14, 1932)

In previous communications evidence has been presented which indicates that an inhibitor is present in transplantable chicken tumors, together with the tumor-producing agent (1). The bases of the assumption that such an inhibitor exists are first, that the removal of a fraction from a tumor extract leaves the tumor agent in a far more active form, and second, that the inhibitor can be extracted from certain tumors in sufficient concentration to neutralize the tumor-producing property of the most active tumor extracts. It has been suggested that the two factors present in the chicken tumor, an agent which causes the malignant transformation of cells and an inhibitor which tends to balance or neutralize this agent, are related to the factors which control the growth and differentiation of normal tissues (2). The tumor agent (Chicken Tumor I) when first studied exhibited a pronounced degree of species specificity, but now shows it to a less extent. On the other hand, many active cell products are not limited in their action to the species producing them. On the basis that the inhibitor from the chicken tumor might be less limited in its effect than the agent, it has been tested on mouse tumors. The results are given in the present paper.<sup>1</sup>

*Methods and Materials.*—The following materials known to neutralize or inhibit the chicken tumor agent were tested on mouse tumors: extracts of desiccated slow-

\*This investigation was carried out under the Rutherford Donation.

<sup>1</sup> A preliminary note on this work has been published (Murphy, Jas. B., and Sturm, E., *Science*, 1931, 74, 180).



growing chicken tumor,<sup>2</sup> exudates from slow-growing tumors, sera from immune chickens, and sera from immunized rabbits (3). As controls to the above tests the following materials, known not to affect the chicken tumor agent, were investigated: extracts of desiccated rapidly growing chicken tumor, exudates from rapidly growing tumor, muscle, brain, and liver from normal chickens, muscle from immune chickens, and normal rabbit and chicken sera. The test solutions were prepared by thoroughly extracting 1 gm. of the tissue desiccates with 30 cc. of water, maintaining the pH at about 7 by the addition of N/10 NaOH. The extracts were then centrifuged to remove the larger particles and the supernatant fluid heated at 52°C. for 30 minutes. The latter procedure was used to destroy the tumor agent in the active extracts and for uniformity the treatment was carried out on all the controls.

The transplantable mouse carcinoma utilized in the experiment was a standard tumor, known as Bashford 63. It usually gives a fairly high percentage of takes and does not often retrogress when once established. The sarcoma principally used is also a standard tumor, known as Crocker 180, characterized by the high percentage of takes it gives in practically all strains of mice, and by the fact that it is not easily influenced by procedures which increase animals' resistance to many of the other transplantable tumors. A third tumor, Mouse Sarcoma S/37, had its origin in the stroma of a transplantable adenocarcinoma, and is notable for its rapidity of growth.

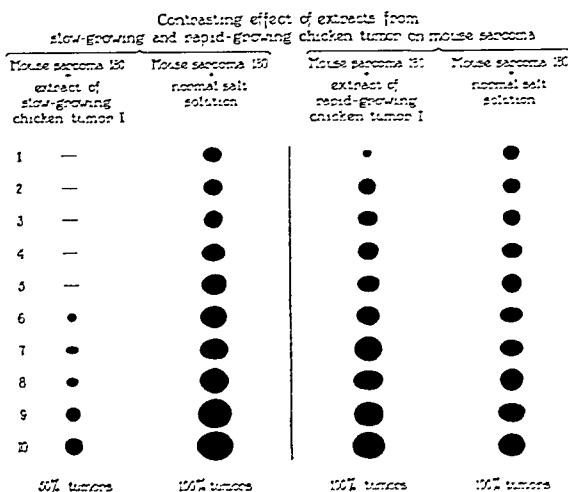
In the test with the carcinoma, grafts of the usual size were cut from the solid part of young tumors; these were placed in the extracts and nicked in several places to give a greater surface of exposure. The controls were immersed in salt solution. The contact was only for the time required to load the grafts into trocars for inoculation. With the sarcomas a suspension was made by forcing the tumors through a fine grill and adding 3 times the volume of normal salt solution. Part of this suspension was mixed with equal amounts of the test extract or fluid and 0.1 cc. inoculated into mice. For the controls the suspensions were diluted with salt solution and equal amounts inoculated. In practically all of the experiments the mice received the test inoculation in one groin and the control in the other, with additional animals inoculated with the control alone.

### *The Effect of Extracts of Chicken Tumor I on Transplantable Mouse Tumors*

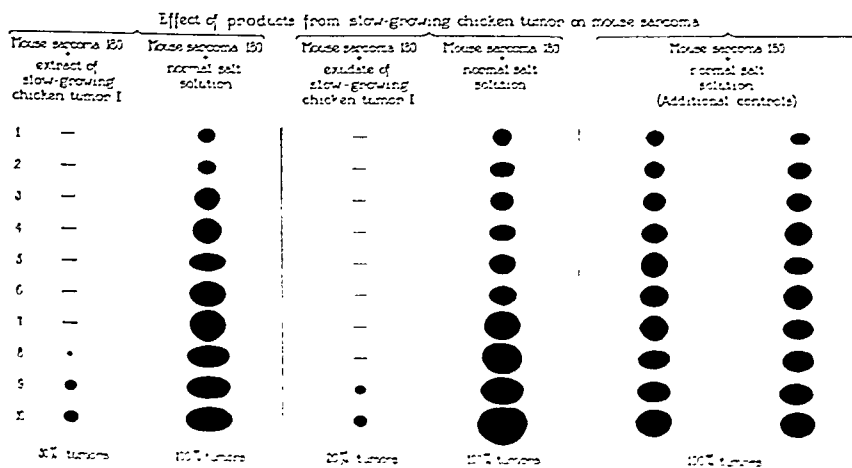
The action of chicken tumor extracts such as are known to inhibit the chicken tumor, and of others without this effect have been princi-

<sup>2</sup> The fact should be emphasized that not all slow-growing examples of Chicken Tumor I yield sufficient inhibitor to have the marked effect reported in this and previous papers. Extracts of desiccates of a large number of tumors were tested and those yielding the greatest concentration of inhibitor were utilized in this test. It is possible that the slow growth rate of some tumors depends on factors other than the presence of an inhibitor in the tumor.

pally tested. So far the investigations have been confined to experiments with the three mouse tumors, for it seemed more important at



TEXT-FIG. 1



TEXT-FIG. 2

the moment to multiply the control tests than to extend the observations to a larger variety of tumors.

*Experiments.*—The average individual experiment was made up of 30 mice divided into groups of 10. Two of these received inoculations of tumor plus a test

fluid in one groin and in the other a control inoculation of the tumor with normal salt solution. The third group was inoculated in both groins with the tumor in

TABLE I

Experiment number	Material inoculated	Number of inoculations	Number negative	Negative <i>per cent</i>	$\chi^2$	<i>P</i>
1	Mouse Tumor 180 plus extract slow C.T.I.	131	102	77.9	126.0	0.000,000
	Mouse Tumor 180 plus salt solution	163	21	12.9		
2	Mouse Tumor 180 plus extract rapid C.T.I.	20	2	10.0	0.6	0.4
	Mouse Tumor 180 plus salt solution	60	3	5.0		
3	Mouse Tumor 180 plus exudate slow C.T.I.	18	16	88.9	61.7	0.000,000
	Mouse Tumor 180 plus salt solution	54	0	0.0		
4	Mouse Tumor 180 plus exudate rapid C.T.I.	20	0	0.0	1.0	0.3
	Mouse Tumor 180 plus salt solution	60	3	5.0		
5	Mouse Tumor 63 plus extract slow C.T.I.	46	10	21.7	0.0	1.0
	Mouse Tumor 63 plus salt solution	87	19	21.8		
6	Mouse Tumor 63 plus exudate slow C.T.I.	17	2	11.8	0.7	0.4
	Mouse Tumor 63 plus salt solution	38	8	21.1		
7	Mouse Tumor 180 plus boiled extract slow C.T.I.	39	11	28.2	0.2	0.65
	Mouse Tumor 180 plus salt solution	50	12	24.0		

salt solution. This use of double controls was done to detect a possible general effect from the local injection of inhibitors. As there was no indication of such

action, the results of the control inoculations, whether in the test animals or in those receiving only control inoculations, are grouped together. In several experiments with materials which failed to show any inhibiting action in two tests the results of the 18 or 20 inoculations were considered sufficient.

The results of two individual experiments are given in Text-figs. 1 and 2. The first contrasts the action on Mouse Tumor 180 of a chicken tumor extract known to inhibit chicken tumors with one

TABLE II

	Number of inoculations	Number negative	Negative <i>per cent</i>
Mouse Tumor 180 plus extract normal muscle..	19	1	5.3
Mouse Tumor 180 plus salt solution.....	32	4	12.5
Mouse Tumor 180 plus immune chicken muscle extract.....	9	1	11.1
Mouse Tumor 180 plus salt solution.....	22	1	4.5
Mouse Tumor 180 plus normal chicken serum..	29	3	10.3
Mouse Tumor 180 plus salt solution.....	60	4	6.7
Mouse Tumor 180 plus immune chicken serum..	19	0	0.0
Mouse Tumor 180 plus salt solution.....	40	1	2.5
Mouse Tumor 180 plus normal rabbit serum...	20	0	0.0
Mouse Tumor 180 plus salt solution.....	40	1	2.5
Mouse Tumor 180 plus immune rabbit serum..	20	0	0.0
Mouse Tumor 180 plus salt solution.....	40	1	2.5

which had no such effect. The second shows the inhibiting action of an extract and an exudate from a slow-growing chicken tumor. The data from all of the experiments, based on over 1000 inoculations, have been brought together in Tables I and II.

In addition to the figures for the tests and controls of each group, we have included an analysis of the principal experiments (Table I) by applying the  $\chi^2$  test with its corresponding probabilities (4). This method tests the independence of the proportionate differences between the two groups under comparison.  $P$  is a measure on the scale of 0 to

fluid in one groin and in the other a control inoculation of the tumor with normal salt solution. The third group was inoculated in both groins with the tumor in

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In addition to the figures for the tests and controls of each group, we have included an analysis of the principal experiments (Table I) by applying the  $\chi^2$  test with its corresponding probabilities (4). This method tests the independence of the proportionate differences between the two groups under comparison.  $P$  is a measure on the scale of 0 to

1 of the probability that the deviations from the theoretical frequencies may be reasonably supposed to be due to the errors of sampling. If  $P$  is below 0.02 we may consider that a real effect had been produced. It will be noted that Experiments 1 and 3 in Table I show an unquestionable difference between the test and the control inoculations, which may be considered proof of an inhibiting action of the extracts and exudates of slow-growing tumors. In Experiments 2 and 4, where the extracts and exudates were obtained from rapidly growing chicken tumors, no inhibiting action is indicated. In Experiment 5 the unusual value of  $P = 1$  was obtained, but undoubtedly the extract in this case had no effect on the Tumor 63. The destruction of the inhibitor by boiling is shown by the results in Group 7.

Table II lists a number of experiments based on smaller numbers, in which tests with extracts from muscle and with normal and immune sera gave negative results.

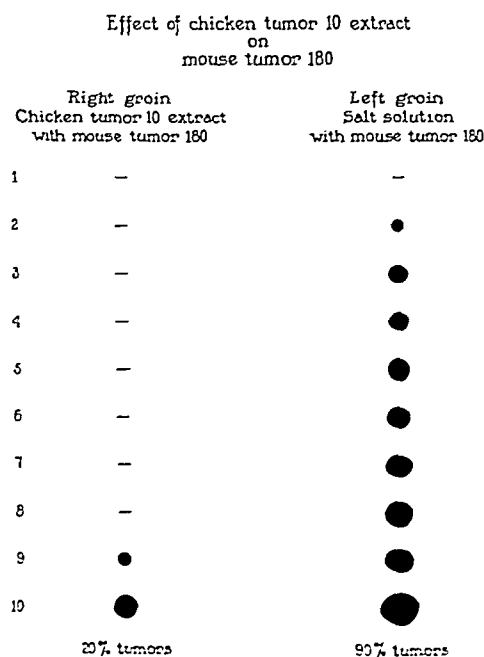
In addition to the experiments included in the tables a few tests were made with extracts of desiccated brain and liver of normal chickens, which were found to be without effect on Mouse Tumor 180. Extensive tests with Mouse Tumor S/37 failed to show any influence on its growth after treatment with extracts known to inhibit chicken tumors and Mouse Tumor 180.

The number of tests is sufficiently large to leave little doubt that the extracts of certain relatively slow-growing chicken tumors and the exudates from such tumors have a definite inhibiting action on a transplantable mouse sarcoma and are without effect on a mouse carcinoma. The number and variety of the controls very largely eliminate the possibility that the result is due to injury from some incidental enzyme or chemical. Perhaps the best indication of this is the failure of products of the rapidly growing tumors to exert any effect.

#### *Effect of Products of Chicken Tumor X on Mouse Tumor*

Andrewes (5) has reported that the serum from chickens bearing either of two slow-growing fibrosarcomas for at least 5 months will neutralize the tumor agents of Chicken Tumor I and the tumor known as Mill Hill 2. He considers this property in the nature of a virus antibody, which would indicate a common or closely related etiologic agent for these tumors. The preceding experiments show that the anti-

bodies developed against the tumor agent either in chickens showing a certain amount of natural resistance or in rabbits actively immunized, while capable of neutralizing the chicken tumor agent, are without effect on the mouse tumors. The possibility that the neutralizing property of the sera described by Andrewes represents the action of an inhibiting factor instead of an antibody has not been eliminated. The following experiments with Chicken Tumor X probably throw some light on the question, as the sera from fowls bearing this tumor were used by Andrewes in his experiments referred to above.



TEXT-FIG. 3

*Experiment.*—Chicken Tumor X has been used as the source of extracts. This tumor, a transplantable fibrosarcoma, derived from a spontaneous tumor, has been under investigation in this laboratory for the last 5 years. As a rule it grows very slowly, often requiring from 8 months to over a year to kill the animal. During this period it attains enormous size. At times it has grown more rapidly but even at these periods metastases have taken place with great rarity. It is transmitted with difficulty by desiccates and only one doubtful result has been obtained with filtrates.



The methods used were the same as those for the preceding group of experiments. The desiccates were prepared from tumors of about a year's growth and the extracts tested on Mouse Tumor 180. The results are presented in Table III and an individual experiment in Text-fig. 3.

It is evident from the figures in Table III, based on five experiments in which 138 inoculations were made, that Chicken Tumor X yields an inhibitor for Mouse Tumor 180. The percentages of complete inhibition are not as striking as those with the inhibitor from Chicken Tumor I. It might have been expected that the inhibitor from the former tumor would be less potent, as it is associated with a tumor agent of relatively low grade activity.

TABLE III

	Number of experiments	Number of inoculations	Number negative	Negative  <i>per cent</i>
Mouse Tumor 180 plus extract of Chicken Tumor X.....	5	69	41	59.5
Mouse Tumor 180 plus salt solution....	5	69	4	5.8

## DISCUSSION

The principal question suggested by the findings is whether the inhibiting action exerted by the extracts of certain desiccated chicken tumors represents a definite, specific force or whether it represents an incidental result, devoid of importance. It is difficult to reconcile the lack of an inhibiting element in extracts from rapidly growing tumors with the latter view. While as yet sufficient evidence is not available for a final conclusion, there are certain facts which justify a tentative interpretation. Perhaps the most important of these is that the inhibitor from a chicken sarcoma acts on a mouse sarcoma and not on a mouse carcinoma; but this observation must be extended to a large variety of tumors before we can accept the reaction as specific. The absence of demonstrable effect of the inhibitor on another mouse sarcoma (S. 37) may be due to the unusual malignancy of this tumor, or there may be some question as to the nature of this growth which is supposed to be a sarcoma but had its origin in the stroma of a carcinoma.

If the inhibitor is a definite factor its possible relationship to anti-

bodies must be considered. The fact that antibodies developed against the chicken tumor have no effect on the mouse sarcoma while inhibitor derived from the tumor does retard these growths, suggests a difference. Andrewes (5) has expressed some doubt that a substance identical with the serum antibody is responsible for the inhibition of the growth of mammalian tumors on the ground that the antibodies seem to act against the filtrate, not against the cells. This point in our opinion requires closer scrutiny, for the inhibitor has but a doubtful effect on the chicken tumor cells and yet does act on mouse tumor cells. We know from the present findings that one of the tumors used in Andrewes' experiments yields an inhibitor for mouse tumors as well as for chicken tumors, and it seems not inconceivable that the sera of fowls bearing the tumor would also contain the inhibiting factor.

It may be suggested tentatively that the property of extracts from certain relatively slow-growing strains of Chicken Tumor I, and Chicken Tumor X, by virtue of which the chicken tumor agents are neutralized and the growth of mouse sarcoma cells is inhibited, represents a definite factor distinct from the usual type of antibody.

#### SUMMARY

Water extracts of desiccates of certain relatively slow-growing strains of Chicken Tumors I and X, or the exudates from such tumors, definitely inhibited the growth of a mouse sarcoma (Crocker 180), and were without effect on a mouse carcinoma (Bashford 63) or Mouse Tumor S/37, a rapidly growing sarcoma derived from the stroma of a carcinoma. Extensive control tests with extracts from rapidly growing chicken tumors, and from tissues of normal and immune chickens showed no inhibiting action. There was no demonstrable action on the mouse tumors of sera from immunized rabbits, which neutralize the chicken tumor agent, nor of the sera from chickens highly immune to the chicken tumors.

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## ACTIVE IMMUNIZATION AGAINST POLIOMYELITIS\*

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(Received for publication, June 23, 1932)

In a previous publication (1), it was shown that for each gram of active cord virus, given intracutaneously as an emulsion, 6 cc. of human convalescent serum, injected subcutaneously, was required to protect an animal from paralysis and that some degree of active immunity followed its use. Immunity, unpreceded by symptoms of the disease, was obtained when the serum was given either at the time of the virus administration, or 3 days prior to it, or 3 days subsequently. However, when virus inoculation followed serum administration, it was probably less effective than when it was given simultaneously with, or before, the injection of serum. Monkeys which received virus at the same time as serum, or before it, produced sufficient immunity for 0.95 cc. of their serum to neutralize 0.05 cc. of 5 per cent cord emulsion, containing more than five completely paralyzing doses of virus. Experiments will be described in this paper which were designed to investigate the production of active immunity by mixtures of virus-containing monkey cord and serum from human convalescents, with or without incubation at 37°C., and of cord without serum. The degree of immunity developed is expressed as the number of completely paralyzing doses of virus neutralized by 0.9 cc. of the serum of the treated animals.

### *Technique*

The technique employed was the same as that described in a previous paper (1). Unless otherwise stated, active cord virus, Fl mixed, glycerinated 3 to 8 weeks, and recently collected human convalescent serum, were used.

\* This research was made possible through a grant received from the Trustees of the Banting Research Foundation, Toronto.

## EXPERIMENTAL

*Experiment 1.*—Each of three animals received mixtures of virus and serum incubated for 2 hours, intracutaneously, Nos. 1-31 and 1-66 receiving 1.5 gm. of cord and 6 cc. of serum while No. 1-65 received 1.5 gm. of cord and 4.5 cc. of serum. Two other animals, Nos. 1-60 and 1-64, received 9 cc. of serum subcutaneously, followed in 3 days by 1.5 gm. of cord, intracutaneously. Monkeys 1-48 and 1-53 received the virus intracutaneously and the serum subcutaneously at the same time; the former was given 1.2 gm. of cord and 7.5 cc. of serum and the latter 1.5 gm. of cord and 9 cc. of serum. Lastly, Monkeys 1-61 and 1-63 received the virus, followed in 3 days by the serum, the former receiving 1.5 gm. of cord and 9 cc. of serum, the latter 1.3 gm. of cord and 7.5 cc. of serum. None of the animals developed any symptoms of poliomyelitis, but three died of intercurrent infection. 2 to 3 months later 0.98 cc. of serum from each animal was tested for protective properties against 0.02 cc. (in Test 1) and against 0.05 cc. (in Test 2) of a 5 per cent emulsion. As the minimal completely paralyzing dose of the virus was 0.01 cc. of 5 per cent emulsion of cord, the tests were made with 2 and 1 M.C.P. minimal completely paralyzing (M.C.P.) doses respectively. One control received incubated mixtures of virus and the serum from Monkey 1-63, which had been given 9 cc. of serum at the time the other animals were inoculated, this being the maximum amount received by the treated animals. A second control received virus and the serum of Monkey 1-48, which had been inoculated with 2 gm. of cord removed from an animal that had been paralyzed 35 days, and whose cord was found, on intracerebral inoculation, to have no infective power.

The course of immunization and the results of the neutralization tests are shown in Table I.

The serum of one animal (No. 1-64), which received serum followed by virus, failed to neutralize, while the serums of the remaining five neutralized 0.02 cc. or 2 M.C.P. doses of virus. Of these five, three serums failed to neutralize while the serum of one monkey (No. 1-63) neutralized 0.05 cc. of cord containing 5 M.C.P. doses of virus. The fifth serum (No. 1-53) either completely, or almost completely, neutralized 5 M.C.P. doses of virus, for it did not prevent the development of symptoms suggestive, but not diagnostic of poliomyelitis. The animal developed irritability, ruffling of the hair and general weakness 24 days later. On the 25th day, although the hind legs appeared weaker than the fore limbs, the cerebrospinal fluid was normal. These manifestations cleared up in 3 to 4 days, and were not diagnostic of poliomyelitis inasmuch as the cerebrospinal fluid was normal and the animal had diarrhea a few days later.

As proven in a previous communication (1), virus following serum

TABLE I

Monkey No.	Cord	Serum	Method	Result	Neutralization Test 1				Neutralization Test 2			
					Serum from Mon-key No.	Cord in 5% emul-sion	Serum	Result	Serum from Mon-key No.	Cord in 5% emul-sion	Serum	Result
1-31	1.5	6	Incubated 2 hrs., injected intracutaneously	Died, intercurrent infection	166	cc.	cc.		166	cc.	cc.	
1-66	1.5	6	" "				0.02	No paralysis				
1-65	1.5	4.5	" "	Died, 5 wks., intercurrent infection								
1-60	1.5	9	Serum subcutaneously. Virus intracutaneously 3 days later	Died, 10 wks.	164	0.02	0.98	No paralysis	166	0.05	0.95	Paralysis, 8 days
1-64	1.5	9	" "									
1-10	1.2	7.5	Virus intracutaneously. Serum subcutaneously, at same time	Died, intercurrent infection, 10 wks.	140	0.02	0.98	No paralysis	164	0.05	0.95	Paralysis, 8 days
1-53	1.5	9	" "		153	0.02	0.98	" "	153	0.05	0.95	*
1-61	1.5	9	Virus intracutaneously. Serum subcutaneously, 3 days later		161	0.02	0.98	" "	161	0.05	0.95	Paralysis, 12 days
1-63	1.3	7.5	" "		163	0.02	0.98	" "	163	0.05	0.95	No paralysis

Monkey No.	Treatment	Serum from Monkey No.	Cord emulsion 5%	Serum	Result
1-18	2 gm. of cord tissue, intracutaneously	—	cc.	cc.	No paralysis
1-63	9 cc. convalescent serum	1-48 1-63	0.005 0.01 0.01	0.995 0.99 0.99	Paralysis, 7 days " " 13 "

\* Possible mild attack of poliomyelitis

administration is less effective than when virus and serum are given simultaneously, or when virus is given before serum. Although Nos. 1-61 and 1-63 were treated in the same manner and although No. 1-61 received more virus than No. 1-63, yet it developed a poorer immunological response to the virus, which suggests that some animals respond to antibody formation better than others.

The antibody is evidently a specific response to the virus, for the control animal (No. 1-48) which received 2 gm. of cord tissue, in which no virus was demonstrable, failed to produce any antibody.

Only one and possibly two out of six animals neutralized 0.05 cc. of 5 per cent cord emulsion containing 5 M.C.P. doses of virus. In other experiments (1), better immunological responses were obtained with smaller quantities of cord and serum, for two out of three monkeys neutralized 0.05 cc. of 5 per cent emulsion, containing more than 5 M.C.P. doses of virus, while the third gave partial neutralization.

The smaller antibody response in these experiments may have been due to a decrease in the activity of the antigen, resulting from a loss of infectivity or to more complete neutralization by the use of a stronger serum. On the other hand, as already pointed out, animals may vary in their immunological responses and so the animals used in Experiment 1, owing to a high incidence of intercurrent infection, may not have responded to the antigen as well as those monkeys used in the previously reported experiments (1). In subsequent experiments information was sought for the elucidation of these possibilities.

*Experiment 2.*—The strength of the serum used in the previously reported experiments, and that being used in the present experiment, were compared by determining the smallest amount of each, which would neutralize 80 M.C.P. doses of virus. The virus, which was titrated at the beginning and at the end of the experiment, maintained its potency.

The results are given in Table II.

It is evident then that the serum used in Experiment 1 had less neutralizing power than the serum used in the previously reported experiments (1), and so the serum was not responsible for reducing the strength of the antigen.

*Experiment 3.*—1 gm. and less of the cord used for the previously reported experiments (1) produced infection when administered intracutaneously. In order

TABLE II

Serum	Test 1		Test 2		Test 3		Test 4		Test 5		Test 6		Test 7	
	Virus emul- sion 5%	Se- rum	Se- rum	Result	Se- rum	Result	Se- rum	Result	Se- rum	Result	Se- rum	Result	Se- rum	Result
Experiment 1	cc. 0.2	0.2	cc. 0.1	No pa- ralysis	cc. 0.05	No pa- ralysis	cc. 0.04	No pa- ralysis	cc. 0.033	Paralysis, 7 days	cc.		cc.	
Previously re- ported ex- periments	0.2	0.2	0.1	" "	0.05	" "	0.04	" "	0.033	No paral- ysis	0.03	No pa- ralysis	0.025	Paralysis, 21 days

Controls				
Monkey No.	Virus emulsion 5%	Result	Monkey No.	Result
2-32	cc. 0.000625	No paralysis	2-94	No paralysis
2-33	0.00125	Paralysis, 23 days	2-93	Paresis right arm
2-34	0.00250	" 10 "	2-96	Paralysis, 6 days
2-16	0.005	" 7 "		

0.2 cc. of 5 per cent virus emulsion = 80 M.C.P. doses of virus.



to determine if the cord being used for the present experiments had equal infectivity, each of five animals received 1 gm. of cord. Four different lots of cord, either as single or pooled specimens, were used. A sixth animal received 1.2, a seventh 1.5 and an eighth 2 gm. of cord.

The results are given in Table III.

At least 2 gm. of cord intracutaneously were necessary to produce infection. Less than this amount failed to infect small young animals as well as older and larger animals. The different cord specimens

TABLE III

Monkey No.	Cord No.	Variations in stage and type of disease	Cord amount <i>gm.</i>	Result
1-93	17, 115, 117, 179, 186, 191	Average incubation period 7.8 days	1	No paralysis
2-00	17, 115, 117, 179, 186, 191	Autopsied immediately after complete paralysis	1	" "
2-09	177, 191, 199, 204, 206	Average incubation period 5.9 days	1	" "
2-23	218	Infected with 80 M.C.P. doses of virus. Long period from onset of paralysis to prostration	1	" "
2-30	218	Autopsied immediately after complete paralysis	1	" "
2-12	188	14 hrs. between complete paralysis and autopsy. Infected with 1 M.C.P. dose of virus	1.2	" "
2-51	218		1.5	" "
2-60	191, 199, 204, 206	Short interval from onset of paralysis to prostration	2	Paralysis, 5 days

were obtained from animals in which the course of the disease varied and from the results obtained it appears that no influence is exerted (1) by long and short incubation periods, (2) by long and short intervals between the onset of paralysis and complete paralysis, (3) by 1 to 14 hours interval between complete paralysis and autopsy, (4) by the animals furnishing the cord specimens receiving 1 or 80 infective doses of virus. Moreover, specimens which had been glycerinated as little as 4 and 19 days respectively, were used, whereas in the earlier experiments the virus had been glycerinated 6 to 16 weeks.

It is obvious then, that at the time of the present experiments, the cord was less infective than that which was used the year previously. Therefore, it is quite possible that the immunological response is a function of cord infectivity and that the weaker cord produced less immunity than the stronger, when used in the same manner.

*Experiment 4.*—The minimal completely paralyzing intracerebral dose of three of these cord specimens (two pooled cords and one single specimen) was deter-

TABLE IV

Pool 1			Pool 2		
Cord specimens 17, 115, 177, 179, 186, 191 Time of glycerination 1-16 mos.			Cords 191, 199, 204, 206 Time of glycerination 2-4 mos.		
Monkey No.	Cord in 5% emulsion	Result	Monkey No.	Cord in 5% emulsion	Result
	cc.			cc.	
2-11	0.00250	No paralysis	2-32	0.000625	No paralysis
*1-68	0.0050	Paralysis, 11 days, left leg	2-33	0.00125	Paralysis, 23 days
2-04	0.01	Paralysis, 5 days	2-34	0.00250	" 10 "
2-05	0.01	" 9 "	2-35	0.005	" 7 "
1-99	0.02	" 6 "			
Cord 218 Time of glycerination 2 mos.			Pool 2 repeated		
2-01	0.000625	No paralysis	2-94	0.00625	No paralysis
2-02	0.00125	" "	*2-93	0.00125	Paresis right arm
2-03	0.0025	Paralysis, 6 days	2-96	0.00250	Paralysis, 6 days

\* Paralysis did not extend.

mined by finding the minimal quantity of each, which would cause a complete paralysis within 24 hours after an inoculation period of less than 13 days in monkeys weighing 2.5 to 4 kilos.

The results are shown in Table IV, where it will be seen that the cord virus of Pool 1, containing specimens which had been glycerinated a considerable time (up to 16 months), produced infection with 0.005 cc., but the minimal completely paralyzing dose was 0.01 cc., and that the M.C.P. dose of Pool 2 and Virus 218 was 0.0025 cc. of a 5 per cent emulsion. Nevertheless, these specimens would not infect in a

dose of 1 gm. of cord intradermally and when accompanied by 6 cc. of convalescent serum gave a poor immunity response. The results of the published experiments (1) have shown that 1 gm. of cord and 6 cc. of serum, in various combinations, produce considerable immunity if the virus in the cord is of such a strength that 1 gm. or less infects, when administered intracutaneously.

*Experiment 5.*—In order to determine the immunological response to a gram of cord of known strength, Monkeys 2-09 and 2-30 each received intracutaneously a gram of Cords 2 and 3 respectively, approximately 8000 M.C.P. doses of virus. Likewise No. 1-93 received a gram of pooled cord Virus 1, approximately 2000 M.C.P. doses of virus. At the same time Monkey 1-75 was given 1 gm. of cord and 6 cc. of serum and No. 1-97 1 gm. of cord and 7 cc. of serum, the cord being given intracutaneously and the serum subcutaneously.

The course of immunization and the results of the neutralization test are shown in Tables V, *a*, and V, *b*.

TABLE V, *a*

Monkey No.	Immunization		Serum	Neutralization Test 1			
	Cord amount	M.C.P. dose virus		Serum from Monkey No.	Cord in 5% emulsion	Serum	Result
	gm.				cc.	cc.	
2-09	1	0.0025	0	2-09	0.01	0.9	No paralysis
2-30	1	0.0025	0	2-30	0.01	0.9	" "
1-93	1	0.01	0	1-93	0.01	0.9	" "
1-97	1	0.01	7				
1-75	1	0.01	6	1-75	0.01	0.9	Paralysis, 12 days
				Convalescent monkey	0.1	0.9	No paralysis

*Controls*

Monkey No.	Cord amount of 5% emulsion	Result
	cc.	
2-40	0.000625	No paralysis
2-51	0.00125	" "
2-52	0.00250	Paralysis, 7 days

TABLE V, b

Neutralization Test 2				Neutralization Test 3				Neutralization Test 4			
Serum of Monkey No.	Cord in 5% emulsion	Serum	Result	Serum of Monkey No.	Cord in 5% emulsion	Serum	Result	Serum of Monkey No.	Cord in 5% emulsion	Serum	Result
2-09	cc. 0.01	0.9	No paralysis	2-09	cc. 0.02	0.9	Paralysis, 12 days	2-09	cc. 0.02	0.9	Paralysis, 11 days
2-30	0.01	0.9	"	2-30	0.02	0.9	No paralysis	2-30	0.03	0.9	" 17 "
1-93	0.01	0.9	"	1-93	0.02	0.9	"	1-93	0.03	0.9	" 11 "
1-97	0.00250	0.9	Paralysis, 5 days								
1-75	0.00250	0.9	Paralysis, 6 days					Convalescent monkey	0.05	0.9	Paralysis, 7 days

*Controls*

Monkey No.	Cord emulsion 5%	Result	Monkey No.	Cord emulsion 5%	Result
3-05	cc.	Paralysis, 6 days	3-08	0.0003125	Paralysis, 23 days
3-04	0.00125	" 6 "	3-07	0.000625	" 6 "
	0.00250		2-83	0.00125	" 6 "

*General Findings*

In neutralization Test 1, the serums of all except No. 1-97 were tested against 0.01 cc. of 5 per cent cord, containing 4 M.C.P. doses of virus. At the same time 0.9 cc. of monkey convalescent serum was tested against 0.1 cc. of 40 M.C.P. doses of virus. The serum of all three animals which received 1 gm. of cord, alone, neutralized 4 M.C.P. doses of virus, while the serum of No. 1-75, which received 6 cc. of serum along with the gram of cord, failed to do so. The monkey convalescent serum neutralized 40 M.C.P. doses of virus.

Tests 2, 3 and 4 were carried out with a cord whose M.C.P. dose is 0.000625 cc. of 5 per cent emulsion. In Test 2 the serums of Nos. 1-97 and 1-75, which received virus and serum, were tested against 0.00250 cc. of 5 per cent cord emulsion, containing 4 M.C.P. doses, while Nos. 2-09, 2-30 and 1-93 were tested with 0.01 cc. or 16 M.C.P. doses. In Test 3, Nos. 2-09, 2-30 and 1-93 were tested against 0.02 cc. or 32 M.C.P. doses of virus, while in Test 4, No. 2-09 was again tested against 0.02 cc., while Nos. 1-93 and 2-30 were tested against 0.03 cc. or 48 M.C.P. doses of virus. The monkey convalescent serum, which neutralized 40 M.C.P. doses of virus in Test 1, was tested against 0.05 cc. or 80 M.C.P. doses of virus in Test 4.

In Test 2, the serum of No. 1-75, which received 1 gm. of cord and 6 cc. of serum, again failed to neutralize 4 M.C.P. doses of virus and, likewise, the serum of No. 1-97, which received 1 gm. of cord and 7 cc. of serum, failed to neutralize the same quantity of virus. The serums of Nos. 2-09, 2-30 and 1-93, each of which received a gram of cord without serum, neutralized 16 M.C.P. doses of virus.

In Test 3, the serum of No. 2-09 failed to neutralize, while the serums of Nos. 2-30 and 1-93 neutralized 32 M.C.P. doses of virus.

In Test 4, the serum of No. 209 again failed to neutralize 32 M.C.P. doses of virus. The serum of No. 1-93 failed to neutralize 48 M.C.P. doses, while that of No. 2-30, in all probability, almost neutralized 48 M.C.P. doses, inasmuch as paralysis took place after a prolonged incubation period (17 days). The specimen of convalescent monkey serum, which, in Test 1, neutralized 40 M.C.P. doses of virus, failed to neutralize 0.05 cc. of cord, containing 80 M.C.P. doses of virus.

The fact that the serum of No. 2-09 neutralized 0.01 cc. and on two

occasions failed to neutralize 0.02 cc. of the same emulsion, points to the accuracy of the neutralization test.

Monkey 2-09 failed to show as much antibody response as No. 2-30, although they both received the same treatment, (1 gm. intracutaneously of a cord whose M.C.P. dose was 0.0025 cc. of a 5 per cent emulsion), indicating, as had already been pointed out in Experiment 1, that animals may vary in their immunological responses.

It is of interest to note that the serum of Monkey 2-30, which received approximately one-half a skin-infective dose, showed nearly as much immunity as a convalescent monkey. The serum of the convalescent monkey neutralized 40 to 80 M.C.P. doses and that of Monkey 2-30 neutralized 32 to 48 M.C.P. doses.

TABLE VI

Monkey No.	M.C.P. dose	Course of immunization		Neutralization in M.C.P. doses of virus
		Cord	Serum	
		gm.	cc.	
2-09	0.0025	1	—	16
2-30	0.0025	1	—	32-48
1-93	0.01	1	—	32
1-97	0.01	1	7	Less than 4
1-75	0.01	1	6	" " 4
Monkey convalescent serum				40-80

These results confirm those of Experiment 1, in showing that the cord in use during these experiments failed to produce much immunological response when used in the proportions of 1 gm. of cord and 6 cc. of serum. The cord without the serum, however, produced considerable immunity. Table VI summarizes the immunological responses to a gram of two specimens of cord, of which the M.C.P. doses were 0.0025 cc. and 0.01 cc. of a 5 per cent emulsion, respectively. The neutralizing power of the serums of these animals is compared with that of convalescent serum.

#### DISCUSSION

The work described in this paper indicates that an appreciable active immunity can be produced with the virus of poliomyelitis in

either of two ways: either by intradermal inoculation with a sub-infective dose of the virus, when it was found that one-half a skin-infective dose conferred immunity; or by various combinations of virus and human convalescent serum, when it was found that one skin-infective dose given intracutaneously, accompanied by a subcutaneous dose of convalescent serum (in the proportions of 0.03 cc. to 80 M.C.P. doses of virus, which proved just sufficient to protect against intracerebral infection) also conferred immunity. The administration by the skin route of the incubated virus-serum mixture, in the same proportions as is innocuous intracerebrally, gave a small degree of immunity. This suggests that the neutralization is incomplete in these proportions; that is to say, some amount of virus remains which is not discernible by intracerebral inoculation in the amounts used, but when 200 times the quantity is inoculated intracutaneously, sufficient virus remains to immunize. The observation that the administration of the serum 3 days previous to the virus prevented immunization and that half a skin-infective dose of virus intracutaneously with one and one-half times the calculated necessary amount of serum subcutaneously, at the same time, or 3 days later than the virus, produced an appreciable immunity, though of lesser degree, indicates that the different routes of administration of serum and virus in different sites play a part in the result obtained. This procedure necessarily prevents the complete action of the serum on the virus and allows of the escape of some virus to immunize the animal. This view is supported by the observation that a subcutaneous dose of one and a half times the amount of serum necessary, allowed the development of measurable though slight immunity. The serum used in this experiment was three-quarters the strength of the other serum used (1) but the proportions were determined by intracerebral protection tests.

It appears then, to immunize effectively with serum-virus mixtures, only just sufficient serum should be used to protect the animal against paralysis.

The known variation in the infectivity of different cord specimens and the known variation in the protective power of individual (2-6), or of pools of convalescent serum (7), requires the titration of each to arrive at a suitably adjusted mixture for immunization.

Since monkey convalescent serum is very much weaker than human

convalescent serum, the detection of immunity in monkeys is liable to be missed unless small amounts of virus are used for test purposes. In the work reported here, it was necessary to use as little as 2 M.C.P. doses of virus (0.00125 cc. of 5 per cent cord suspension) and in strong immunity 32 M.C.P. doses of virus (0.02 cc. of 5 per cent cord suspension) was as much as was neutralized by 0.9 cc. of monkey serum. With human serum, on the average, 80 M.C.P. doses of virus (0.1 cc. of 5 per cent cord suspension) are neutralized by 0.04 cc. of serum (7). It is possible that other workers (8, 9, and others) have failed to detect immunity in monkeys by requiring the neutralization of too large a dose of virus, expecting something comparable to the human conditions.

#### CONCLUSIONS

1. A single intracutaneous inoculation with a subinfective dose of the virus of poliomyelitis produces considerable immunity.
2. Virus-serum combinations produce an appreciable immunity, providing just sufficient serum is used to protect the animal from paralysis. If there is an excess of serum, the degree of immunity is considerably reduced.

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## A COMPARISON BETWEEN CONVALESCENT SERUM AND NON-CONVALESCENT SERUM IN POLIOMYELITIS\*

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The work of Anderson and Frost (1), Peabody, Draper and Dochez (2), Leake (3), Aycock and Kramer (4) and others has shown that the serum of so called normal individuals, who have had no clinical history of poliomyelitis, may neutralize the virus of poliomyelitis. Aycock and Kramer (4), Schultz and Gebhardt (5), found this specific neutralizing power in the serum of most urban adults.

On the grounds that the majority of urban adults are thought to be immune to poliomyelitis, tests had been made for the presence of neutralizing substances in the sera of twenty-nine adults, thirteen of whom had a history of contact with cases of poliomyelitis and all of whom denied a history of clinical symptoms. At the same time, the potency of the serums was compared with that of pooled convalescent serums.

Anderson and Frost (1) were the first to demonstrate neutralizing substance for the virus of poliomyelitis in the serums of non-convalescent adults and Peabody, Draper and Dochez (2), in their studies, found four out of six serums, obtained from individuals with no clinical history of the disease, neutralized the virus. Later Leake (3) made similar observations, but the first extensive study in this direction was undertaken by Aycock and Kramer (4) who, in their series of urban residents, found 69.6 per cent with neutralizing substance and that the serums of 87.5 per cent of the adults of this group neutralized the virus. Likewise, Schultz and Gebhardt (5) showed that, of thirteen so called normal adult serums, 69.1 per cent neutralized the virus of poliomyelitis. Others (6-9) have also demonstrated specific neutralizing substances for the virus of poliomyelitis in the blood serum of non-convalescent adults.

Although the neutralizing power of these non-convalescent serums has not been

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compared with batches of convalescent serum, Shaughnessy, Harmon and Gordon (7) claim that the neutralizing power of serums from normal individuals may equal, or exceed, that of individual convalescent serum. On the other hand, Weyer, Park and Banzhaf (8) did not find any of six adult serums to equal the neutralizing power of either of two individual convalescent serums.

### *Technique*

Cord virus which had been glycerinated 2 to 4 months was used. In order to obtain a fairly even distribution of virus, six or seven segments of each cord specimen were used.

The neutralization test was carried out as follows: 1 cc. of a 5 per cent cord emulsion and 1 cc. of serum were incubated 2 hours (with frequent agitation) and then, after a second period of 8 hours on ice, the mixture was diluted to 5 cc., 1 cc. of which was injected into the frontal lobe of a monkey. In the first test, 1 cc. of undiluted serum was added to 1 cc. of virus, but in the succeeding tests, 1 cc. of serum, diluted 1:2, 1:3, 1:4 and so forth, was added.

### EXPERIMENTAL

The average level of neutralizing power of convalescent serum was determined by finding the smallest quantity of each of four batches of serum which would neutralize 80 minimal completely paralyzing (M.C.P.) doses of virus and then dividing the sum of these amounts by four. For the purposes of this work, the M.C.P. dose is limited to the smallest quantity of virus-containing tissue that will cause a complete and rapid paralysis in monkeys weighing 2.5 to 4 kilos, within 13 days. In my experience, less than a completely paralyzing dose either failed to produce symptoms, or at most produced paralysis after a prolonged incubation period. More than a completely paralyzing dose invariably produced severe paralysis. The constancy of these results leads me to believe that monkeys do not vary greatly in their individual susceptibility to the disease.

*Experiment 1.*—The separate cords of three monkeys and a pool of cords from four monkeys, all removed at the height of paralysis and preserved in glycerine 2 to 4 months, were titrated in order to determine their M.C.P. dose (Table I). Each gave approximately the same M.C.P. dose, which seems to indicate a remarkable constancy, for cord infected with this virus at least, at that stage of the disease. After a further period of 2½ months, the pooled specimen did not show any change in its infectivity.

*Experiment 2.*—To determine the average neutralizing power of convalescent serum, the virus used to test the convalescent serums was a pooled specimen of

six segments from each of four separate cords and on the above evidence constant results were expected. The virus emulsion, made up from cord tissue of each of these twenty-four segments, was titrated at the beginning and at the completion of the experiments and showed no appreciable change in its strength.

Each batch of pooled convalescent serum represented that of twelve to twenty-eight donors, who had had the disease at least 2 years previously. The results are given in Table II.

TABLE I  
*Titration of Four Specimens of Cord*

(1) Glycerinated Mar. 15, 1931 Titration July 15, 1931 Incubation period—6 days			Pooled specimen		
			(4) Glycerinated June and July, 1931 Titration Sept. 1, 1931 Incubation period—5-7 days		
Monkey No.	Cord	Result	Monkey No.	Cord	Result
	mg.			mg.	
1	0.0625	No paralysis	1	0.03125	No paralysis
2	0.125	Paralysis, 13 days	2	0.0625	Paralysis, 23 days, slow course
3	0.250	" 9 "	3	0.125	Paralysis, 10 days
4	0.5	" 7 "	4	0.250	" 7 "
5	1.0	" 7 "			
(2) Glycerinated July 29, 1931 Titration Sept. 18, 1931 Incubation period—13 days			(4) 2½ mos. later		
1	0.03125	No paralysis	1	0.03125	No paralysis
2	0.0625	" "	2	0.0625	Paresis right arm
3	0.125	Paralysis, 7 days	3	0.125	Paralysis, 6 days
(3) Glycerinated Aug. 7, 1931 Titrated Oct. 13, 1931 Incubation period—7 days					
1	0.03125	No paralysis			
2	0.0625	" "			
3	0.125	Paralysis, 7 days			

Averaging the titrated neutralizing power of these four specimens of pooled convalescent serum, representing over a hundred donors, a mean of 0.04 cc. neutralizing 80 M.C.P. doses of virus is obtained. The batches of serum varied considerably in strength, for the weakest showed only 58 per cent of the neutralizing power of the strongest and the remaining two approximated to the arithmetical average figure.

TABLE II

Seri	Cord	Test 1		Test 2		Test 3		Test 4		Test 5		Test 6		Test 7	
		Se- rum	Result	Se- rum	Result	Se- rum	Result	Se- rum	Result	Se- rum	Result	Se- rum	Result	Se- rum	Result
		mg.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
1	10	0.2	No paralysis	0.1	No paralysis	0.05	No paralysis	0.04	No paralysis	0.033	No paralysis	0.029	No paralysis	0.025	Paralysis, 21 days
2	10	0.2	"	0.1	"	0.05	"	0.04	"	0.033	Paralysis, 7 days				
3	10	0.2	"	0.1	"	0.05	"	0.04	"	0.033	Paralysis, 11 days				
4	10	0.2	"	0.1	"	0.05	"	0.04	Paralysis, 8 days						

## Controls

Monkey No.	Cord	Result		Monkey No.	Cord	Result	
		mg.	cc.			mg.	cc.
2-32	0.03125	No paralysis Paralysis, 23 days, slow " 10 " rapid " 7 " "		2-94	0.03125	No paralysis Paresis right arm Paralysis, 6 days, rapid	
2-33	0.0625			2-93	0.0625		
2-34	0.125			2-96	0.125		
2-46	0.250						

10 mg. of cord emulsion contains 80 m.c.p. doses.

*Experiment 3. The Titration of Normal Adult Serums.*—The adult serums were tested by the technique used in titrating convalescent serum. A similar quantity of the same pooled virus was used and the tests were carried out at the same time, so that, when neutralizing substances were demonstrated, the titre could be compared with that of the convalescent serums. Of the twenty-nine adults tested, fourteen had a history of known contact with cases of poliomyelitis, but, in view of the recent work of Kramer and Aycock (10), as one was exposed but 2 weeks prior to the tests, he was listed as a non-contact.

In addition to titrating the virus at the beginning of the experiment and at the time of the last completed set of neutralization tests, an additional control monkey received a mixture of swine serum and virus, in order to show that the serum of an animal, said to be refractory to the virus of poliomyelitis (11), did not contain specific neutralizing substances. The results are given in Table III.

In the first test twenty-two or 76 per cent neutralized the virus, so that, if the protective power is expressed in terms of the average protective power of convalescent serum, seven contained less than two-fifths the neutralizing power of the average for the four convalescent serums. It is possible that in two cases, (Nos. 2 and 19), the virus was partially neutralized by the serums, for the disease ran a much slower course than usual, after incubation periods of 12 and 13 days, respectively.

In the second test nineteen or 65 per cent neutralized, so that three were below three-fifths strength, but equal to two-fifths the strength of convalescent serum. In the third test, two failed to neutralize, and so contained less than four-fifths but equalled three-fifths the strength of convalescent serum, so that seventeen had at least four-fifths the protective power of the average for the four pooled convalescent serums. Of these seventeen, six out of the ten which were tested, equalled the neutralizing power of convalescent serum.

The pooled serum of non-contacts had at least two-fifths but less than three-fifths the average neutralizing power of convalescent serum and the pooled serum of contacts had at least three-fifths but less than four-fifths the average neutralizing power of convalescent serum.

*Experiment 4.*—The seven serums which had not been worked out in Experiment 3, which had at least four-fifths the neutralizing power of convalescent serum, were tested to determine whether or not they equalled the neutralizing power of convalescent serum. In addition, six of the normal serums which at

TABLE III

Blood donor No.	Age	History of contact	Test 1		Test 2		Test 3		Test 4	
			Cord emul-sion	Se-rum	Result	Se-rum	Result	Se-rum	Result	Se-rum
	yr.		mg.	cc.		cc.		cc.		cc.
1	31	None	10	0.1	Paralysis, 8 days					
2	42	"	10	0.1	" 12 "					
3	27	"	10	0.1	" 12 "					
4	35	"	10	0.1	No paralysis	0.07	Paralysis, 13 days			
5	24	"	10	0.1	" "	0.07	" 14 "			
6	41	"	10	0.1	" "	0.07	No paralysis	0.05	Paralysis, 13 days	
7	33	"	10	0.1	" "	0.07	" "	0.05	" 8 "	
8	15	"	10	0.1	" "	0.07	" "	0.05	No paralysis	0.04
9	21	"	10	0.1	" "	0.07	" "	0.05	" "	0.04
10	21	"	10	0.1	" "	0.07	" "	0.05	" "	Not complete
11	23	"	10	0.1	" "	0.07	" "	0.05	" "	" "
12	23	"	10	0.1	" "	0.07	" "	0.05	" "	" "
13	26	"	10	0.1	" "	0.07	" "	0.05	" "	" "
14	19	"	10	0.1	" "	0.07	" "	0.05	" "	No paralysis
15	21	"	10	0.1	" "	0.07	" "	0.05	" "	" "
16	19	2 wks.	10	0.1	Paralysis, 11 days					
17	54	Yes	10	0.1	" 8 "					
18	30	2-3 mos.	10	0.1	" 11 "					
19	32	2-3 " and previously	10	0.1	" 13 "					
20	30	2-3 "	10	0.1	No paralysis	0.07	(Slow course) Paralysis, 12 days			
21	37	2-3 " and previously	10	0.1	" "	0.07	No paralysis	0.05	No paralysis	0.04
22	28	2-3 " "	10	0.1	" "	0.07	" "	0.05	" "	0.04
23	35	6-8 wks.	10	0.1	" "	0.07	" "	0.05	" "	Not complete
24	24	2 mos. and previously	10	0.1	" "	0.07	" "	0.05	" "	" "

25	30	2-3 mos.	10	0.1	No paralysis	0.07	No paralysis	0.05	No paralysis	0.04	Not complete
26	25	3 mos. and previously	10	0.1	"	0.07	"	0.05	"	0.04	No paralysis
27	28	3 " "	10	0.1	"	0.07	"	0.05	"	0.04	"
28	31	Experimental disease	10	0.1	"	0.07	"	0.05	"	0.04	"
29	35	"	10	0.1	"	0.07	"	0.05	"	0.04	"
		Pooled contacts	10	0.1	"	0.07	"	0.05	Paralysis, 13 days		
		Pooled non-contacts	10	0.1	"	0.07	Paralysis, 21 days	0.05			

Controls

Monkey No.	Cord emulsion	Result	Monkey No.	Cord emulsion	Result
2-32	0.03125	No paralysis	2-96	0.03125	No paralysis
2-33	0.0625	Paralysis, 23 days, slow course	2-93	0.0625	Paresis right arm
2-34	0.125	" 10 " rapid course	2-94	0.125	Paralysis, 6 days, rapid course

One monkey received 0.25 mg. of cord and 0.9 cc. of swine serum—paralysis 7 days. 10 mg. of cord = 80 M.C.P. doses.



TABLE IV

Blood donor No.	Age	History of contact	Cord emulsion	Test 4		Test 5	
				Serum	Result	Serum	Result
	yrs.		mg.	cc.		cc.	
10	21	None	2.5	0.04	Paralysis, 5 days		
11	23	"	2.5	0.04	" 12 "		
12	23	"	2.5	0.04	No paralysis		
13	26	"	2.5	0.04	" "	0.033	Paralysis, 8 days
14	19	"	Neutralized in last experiment			0.033	" 11 "
15	21	"	"	"	" " "	0.033	No paralysis
23	35	6-8 wks.	2.5	0.04	Paralysis, 10 days		
24	24	2 mos. and previously	2.5	0.04	" 6 "		
25	30	2-3 mos.	2.5	0.04	No paralysis		
27	28	3 mos. and previously	Neutralized in last experiment			0.033	Paralysis, 11 days
28	31	Experimental disease	"	"	" "	0.033	" 15 "
29	35	" "	"	"	" "	0.033	No paralysis
Convalescent Pool 5			2.5	0.04	Neutralized	0.033	Paralysis, 30 days

*Controls*

Monkey No.	Cord emulsion	Result	Monkey No.	Cord emulsion	Result
	mg.			mg.	
3-05	0.0625	Paralysis, 6 days	3-08	0.015625	Paralysis, 23 days
3-04	0.125	" 6 "	3-07	0.03125	" 6 "
3-02	0.250	" 7 "	2-83	0.0625	" 6 "
			2-62	0.125	" 6 "
			2-65	0.125	" 5 "

2.5 mg. of cord = 80 M.C.P. doses.

least equalled the average potency of the convalescent serums were selected and tested further. As an additional comparison, a fifth sample of pooled convalescent serum was included in the experiment.

The virus used in this experiment had four times the infectivity of that used in the previous experiment, the M.C.P. dose being 0.03125 mg., so that 80 M.C.P. doses were represented in 2.5 mg. of cord. The results are shown in Table IV.

Convalescent serum, Pool 5, containing the serums of twenty donors, had the same neutralizing power as the average of the other four pools.

Of the seven serums, which showed in the last test at least four-fifths the neutralizing power of convalescent serum, only three equalled the neutralizing power of convalescent serum. Combining the results of Experiments 3 and 4, nine out of the twenty-nine serums tested at least equalled the neutralizing power of convalescent serum. Of the six of these tested further (Test 5), two showed higher neutralizing power than the average of the convalescent serums.

The results may be expressed as follows:

Potencies of 7 serums were below 2/5 the average of the convalescent serums

3	"	equalled	2/5	"	"	"	"	"	"
2	"	"	3/5	"	"	"	"	"	"
8	"	"	4/5	"	"	"	"	"	"
9	"	at least equalled	"	"	"	"	"	"	"

#### Summary

In 7 cases 0.1 cc. of serum failed to neutralize 80 m.c.p. doses of virus

" 3	"	0.07	"	"	"	"	"	80	"	"	"	"
" 2	"	0.05	"	"	"	"	"	80	"	"	"	"
" 8	"	0.04	"	"	"	"	"	80	"	"	"	"
" 9	"	0.04	"	"	"	neutralized		80	"	"	"	"

Two out of six of these nine showed higher neutralizing power since 0.033 cc. of serum neutralized 80 m.c.p. doses of virus and five pooled convalescent serums gave an average figure of 0.04 cc. of serum neutralizing 80 m.c.p. doses of virus.

#### DISCUSSION

Of twenty-nine serums, the largest series of non-convalescent urban adults tested for antibody, twenty-two or 76 per cent showed definite protective power against poliomyelitis virus. Two other serums appeared to give partial neutralization, since in the animals receiving these serums the disease ran a much slower course after incubation periods of 12 and 13 days, respectively; therefore, in this series, the incidence of specific neutralizing substances against the virus of poliomyelitis, is approximately 80 per cent, which agrees with the figures obtained by Aycock and Kramer (4).

Out of 16 non-contacts tested, 12 had at least two-fifths, 10 had at least three-fifths, 8 had at least four-fifths and 4 had at least equal the average of the neutralizing power of the convalescent serums. Of the 13 contacts tested, 10 had at least two-fifths, 9 had at least three-fifths, 9 had at least four-fifths and 5 had at least equal the neutralizing power of convalescent serum. In this series then, known contacts and

avowed non-contacts were equally represented in the serums of low potency, but in the serums of higher potency the known contacts predominated slightly. The pool of the serums of known contacts was of slightly higher value than the pool of the serums of non-contacts, but they were respectively a little above and a little below half the strength of the average obtained for pooled convalescent serum.

Shaughnessy *et al.* (7), comparing individual convalescent and so called normal adult serums, claimed that the latter had as a rule better neutralizing power than the former. It is quite likely, however, that, in many of their neutralization tests, the virus tissue used was not active, since eight of fourteen controls did not contract paralysis with as much as 1 cc. of a 5 per cent emulsion (12). This widespread distribution of neutralizing substances amongst adults and the high titre in more than half of them might result either from exposure to the virus of poliomyelitis, and subsequent immunity through a subclinical attack of the disease, or it may occur as a natural maturation with age. There is considerable epidemiological and experimental evidence to suppose that the former mechanism may be partly responsible for the development of the neutralizing substances in these adult serums. Frost (13), who first suggested this hypothesis, emphasized the differences in the age distribution of the disease in urban and rural districts and concluded that an immunity, which was directly proportional to concentration of population, developed with age. In addition, Aycock (14) has shown that the age distribution is similar to that of measles and diphtheria and believes that subclinical infection accounts for the widespread immunity in adults against the virus of poliomyelitis. As further evidence it has been shown that the incidence of neutralizing substance against the virus of poliomyelitis parallels the Schick test and that the incidence of this neutralizing substance increases with age according to concentration of population, (Aycock and Kramer (4)).

The work here described confirms the high incidence of antibody amongst urban adults and shows that a good proportion have a high degree of neutralizing ability. It has also been shown that the incidence of more potent serum is greater amongst contacts, the majority of whom had frequent exposure to the virus, than amongst non-contacts, which supports the hypothesis that exposure is a factor in bringing about immunity.

*The Clinical Use of This Serum*

To meet an emergency, serum was collected, for therapeutic purposes, from eleven of the seventeen normal persons showing high protective power in their serum, on the grounds that these serums seemed to be quite as strong as the average convalescent serum, and that larger quantities could be obtained from adults than from convalescent patients, the majority of whom are children.

Over 8 litres of blood was collected and the pooled serum was used in the treatment of sixteen early cases which occurred at the height of the epidemic. In Table V a comparison is made between these and twenty-five cases, which occurred at approximately the same time and which were treated with convalescent serum.

TABLE V

Serum	No. cases	Total serum	No paralysis		Mild paralysis		Severe paralysis		Deaths	
			No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
Convalescent	25	cc. 21-25 4-50	20	80	2	8	3	12	1	4
Selected adult	16	7-25 9-50	13	81.2	0	0	3	18.8	1	6.3

The series is small and difficult to interpret, but the rapid fall of temperature and the amelioration of symptoms, in the cases which did not develop paralysis, was equally evident with the serum from both sources.

Three cases which developed paralysis following non-convalescent serum and five which contracted paralysis after the administration of convalescent serum, did not show any fall of pulse or temperature or any symptomatic improvement after the intravenous administration of serum.

In these early cases of poliomyelitis, there is a similarity in the responses to convalescent and high titre non-convalescent serums. Zingher (15) reported satisfactory results, for a small series of pre-paralytic poliomyelitis patients, with large quantities of pooled untested serum from normal adults.

The supply of convalescent serum, children being the usual source,

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The supply of convalescent serum, children being the usual source,

will not always meet demands in an epidemic. The present observations indicate that the supply can be considerably augmented by serum of tested adults. Furthermore, by obtaining serum from tested adults, the donor list is reduced and there is a considerable amount of time saved. In order to obtain 13.5 litres of convalescent serum for the 1931 epidemic in Montreal, 200 bleedings were necessary and a donor list of 150 persons was used.

#### CONCLUSIONS

1. Of twenty-nine so called normal urban adults tested, seven had less than two-fifths, three had two-fifths, two had three-fifths, eight had four-fifths and nine had a neutralizing power at least equal to the average of five batches of pooled convalescent serum.

2. Known contacts and avowed non-contacts were equally represented in the serums of low potency, but in the serums of higher potency, the known contacts predominated.

3. The pooled serums of known contacts and of non-contacts were respectively a little above and a little below half the strength of the average obtained for pooled convalescent serum.

4. Two series of early cases of poliomyelitis, the one treated with normal serum of proved protective power, the other with convalescent serum, showed no advantage of one type of serum over the other.

I wish to thank Professor E. G. D. Murray for his advice and suggestions throughout the course of this work, and the Staff of the Children's Memorial Hospital for their kindness in making these clinical studies possible.

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STUDIES ON THE QUANTITATIVE ACTION OF A SPECIFIC  
ENZYME IN TYPE III PNEUMOCOCCUS DERMAL  
INFECTION IN RABBITS

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Avery and Dubos (1-3) have reported the isolation of a bacterium capable of specifically decomposing the capsular polysaccharide of Pneumococcus Type III. This decomposition was shown to be due to the action of an intracellular enzyme which could be extracted in active form from the bacilli. Potent preparations of this enzyme protected mice against infection with virulent Type III Pneumococcus and exerted a curative action on an infection already established.

Recently we have reported that this enzyme, when used in sufficient amounts and under stated conditions, was capable of bringing about a favorable and early termination of the experimental disease induced by infecting rabbits intradermally with Type III Pneumococcus (4).

A strain of Type III Pneumococcus which was especially virulent for rabbits was used in producing the characteristic infection described in detail in previous papers (5-8). An edematous lesion makes its appearance at the point of inoculation in the skin of the rabbit's flank and rapidly spreads ventrally, creating within 15 to 20 hours a widespread lesion most pronounced in the dependent portions of the skin. With the development of the local lesion there is a rapid elevation of body temperature and this remains at high levels until death or eventual recovery. Pneumococci escape from the lesion into the general circulation and create a bacteriemia of varying but generally increasing severity. Following the intravenous injection of large quantities of enzyme 24 hours after infective inoculation, a large majority of the animals survived. The injection of the enzyme is followed immediately by the disappearance of organisms from the blood stream. The temperature at first rises but within a few hours begins to fall and normal levels are generally reached within 24 hours. With the subsidence of the bacteriemia and the fall in temperature the local lesion begins to show signs of healing and the skin eventually returns to its normal state. Successful experiments were also reported in which smaller amounts of enzyme were injected repeatedly over a period of days.

This earlier work was chiefly concerned with the qualitative aspects of the prob-

lem. However, the results indicated that large quantities of enzyme appeared to be necessary for successful therapy in rabbits which had a high bacteriemia while smaller amounts seemed adequate in animals having a lesser number of organisms in the blood at the time of treatment.

The experiments reported in the present paper deal with the quantitative factors involved in the action of the specific enzyme in this experimental disease.

#### EXPERIMENTAL

The general plan of these experiments was to give each infected rabbit one intravenous injection of a determined amount of enzyme 24 hours after infective inoculation. For the most part the experimental methods were the same as those reported in the previous paper (4).

*Culture.*—The rabbit-virulent strain of Type III Pneumococcus was maintained in rabbit blood broth with frequent animal passage. Virulence for rabbits was such that 0.000,01 cc., given intradermally, caused death or a protracted disease of severe character.

*Infection.*—Healthy male rabbits weighing from 1,800 to 2,000 gm. were used. Animals were injected intradermally, at a site midway on the flank area, with 0.2 cc. of a dilution of the culture containing the desired number of organisms. In this series certain of the animals received 0.001 cc. of culture; others, for reasons to be mentioned below, received 0.2 cc.

*Enzyme Preparations.*—The enzyme preparations used in these experiments were, for the larger part, purified and concentrated lots prepared by the method described by Dubos (9). The potency was carefully determined by the method previously described.

*Enzyme Injections.*—Each treated animal received a single injection of enzyme 24 hours after infective inoculation. The desired amount of the enzyme preparation was warmed to 37°C., and injected intravenously at a rate not greater than 1.5 cc. per minute.

*Blood Cultures.*—The number of organisms per cc. of blood was determined by a procedure previously described in detail (5). In brief, the method consists in plating 0.4 cc. of blood taken from the marginal ear vein with an accurately graduated syringe. It is obvious that this method is open to many experimental errors and that counts so obtained are not entirely accurate. An analysis of the results, however, shows that they are relatively significant.

The estimation of the number of pneumococci in the circulating blood takes no account of the number in the lesion. This cannot be readily determined. If the edema fluid is removed and cultured, the

number of pneumococci present is usually found not to be extremely large. However, a large number may be present in the tissues. The number in the lesion undoubtedly varies within limits depending upon the amount of the tissue involved, and possibly upon other unknown factors.

Dubos and Avery (2) have shown by experiments *in vitro* that, after definite incubation period, the total amount of specific substrate decomposed bears a quantitative relationship to the concentration and activity of the enzyme preparation used. If it were possible to apply this finding to experimental infections, the minimal amount of enzyme necessary to bring about recovery should bear a quantitative relationship to the amount of specific capsular polysaccharide in the animal body. This substance is of course associated with the pneumococci but may also be present in a free form entirely detached from the bacteria. It is technically impossible to determine the total amount of the specific polysaccharide in the infected animal or even to estimate the total number of pneumococci. The nearest approximation is the determination of the number of viable pneumococci in the circulating blood. Other factors being the same, the degree of blood infection should, on theoretical grounds, bear some quantitative relationship to the amount of enzyme necessary to bring about recovery. When no invasion of the blood has occurred this relationship, of course, would not hold.

As the experimental work progressed it became apparent that the amount of enzyme necessary to bring about recovery was definitely related to the number of organisms in the blood stream. It therefore became desirable to study a large number of animals showing wide variations in the degree of blood infection. With an infective amount of 0.001 cc. of culture there was considerable individual variation in the animals, the number of organisms per cc. of circulating blood varying from 5 to 10,000, the higher numbers being exceptional. In order to insure an early and massive blood invasion the infective inoculum was increased to 0.2 cc., an amount 200 times greater than that previously used. This gave rise to an infection which differed from the previous one only in that it was much more rapidly fatal if untreated and in that the numbers of pneumococci per cc. of blood were relatively enormous, frequently too great to estimate by the plate method. In

the series of animals reported in this paper about one-half have been infected with 0.2 cc. and one-half with 0.001 cc. of culture.

An apparently insurmountable difficulty was that at the time of treatment there was no knowledge of the number of pneumococci in the blood stream. This information is necessarily not available until 24 hours thereafter. In the hope of finding some index of the bacteriemia which might be available at once, we have compared the number of organisms in the blood stream with the height of the temperature, the temperature trend, the leukocyte count, the trend of this count, the appearance and magnitude of the lesion, and the weight trend. No satisfactory correlation was obtained with any of these factors. Because of this fact the amounts of enzyme given have been arbitrarily chosen.

### *The Amount of Enzyme and the Outcome of the Disease*

We have now studied a series of 85 rabbits of which 61 have been treated with a definite amount of enzyme 24 hours after infective inoculation. The others have served as controls. The data on this series of animals are shown in Table I. The animals have been arranged in groups depending on the numbers of organisms in the blood stream at the time of treatment. Thus in Group A are placed the animals in which the blood cultures were negative; in Group B, those with 3 to 10 organisms per cc.; etc. In each group the animals are arranged in series according to the amounts of enzyme each received. In each instance the degree of bacteriemia and the results of the treatment are indicated.

*Group A.*—Of six untreated controls, one survived. Among the treated animals there were scattering deaths until the amount of enzyme used was greater than 2.5 units. Even in these fatal cases, however, death occurred, on an average, over 4 days later than in the controls. When 3 or more units were used, all animals survived.

*Group B.*—In this group are included those animals in which the blood cultures showed from 3 to 10 organisms per cc. The smallest amount of enzyme used in this group was 2.5 units. All these animals survived whereas the untreated controls died.

*Group C.*—In the animals in this group, at the time of treatment, 11 to 100 organisms per cc. of blood were found. An apparent irregularity is caused by the fact that one animal died after receiving 10 units of enzyme whereas another survived after only 6.6 units. However, it should be noted that the bacteriemia was much higher in the former animal. The minimal amount of enzyme efficacious in this group, therefore, was between 10 and 20 units.

TABLE I

*Results of the Administration of Various Amounts of Specific Enzyme in Infected Rabbits Having Varying Numbers of Type III Pneumococci per Cc. of Blood*

Determined amounts of enzyme preparations injected intravenously into rabbits 24 hrs. after infective inoculation with Type III Pneumococcus

Group	Rabbit	Bacteriemia at 24 hrs.	Units of enzyme	Result
A	1	Negative	None	D 114
	2	"	"	" 88
	3	"	"	" 70
	4	"	"	" 64
	5	"	"	" 53
	6	"	"	S
	7	"	1	"
	8	"	1.25	"
	9	"	2	D 162
	10	"	2	S
	11	"	2.5	D 192
	12	"	2.5	" 190
	13	"	3	S
	14	"	3	"
	15	"	6.6	"
	16	"	10	"
	17	"	20	"
	18	"	100	"
	19	"	100	"
	20	"	100	"
B	1	10	None	D 84
	2	5	"	" 84
	3	5	"	" 51
	4	3	"	" 150
	5	3	2.5	S
	6	3	6.6	"
	7	3	10	"
	8	3	17.5	"
	9	10	20	"
	10	10	50	"
	11	5	100	"
	12	8	100	"
	13	10	100	"
	14	10	100	"
C	1	85	None	D 111
	2	78	"	" 36
	3	52	"	" 55
	4	40	"	" 52

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	3	"	"	" 70
	4	"	"	" 64
	5	"	"	" 53
	6	"	"	S
	7	"	1	"
	8	"	1.25	"
	9	"	2	D 162
	10	"	2	S
	11	"	2.5	D 192
	12	"	2.5	" 190
	13	"	3	S
	14	"	3	"
	15	"	6.6	"
	16	"	10	"
	17	"	20	"
	18	"	100	"
	19	"	100	"
	20	"	100	"
B	1	10	None	D 84
	2	5	"	" 84
	3	5	"	" 51
	4	3	"	" 150
	5	3	2.5	S
	6	3	6.6	"
	7	3	10	"
	8	3	17.5	"
	9	10	20	"
	10	10	50	"
	11	5	100	"
	12	8	100	"
	13	10	100	"
	14	10	100	"
C	1	85	None	D 111
	2	78	"	" 36
	3	52	"	" 55
	4	40	"	" 52



TABLE I—*Continued*

Determined amounts of enzyme preparations injected intravenously into rabbits 24 hrs. after infective inoculation with Type III Pneumococcus

Group	Rabbit	Bacteriemia at 24 hrs.	Units of enzyme	Result
<i>C—concluded</i>	5	13	None	D 62
	6	23	3	" 40
	7	20	3	" 108
	8	15	6.6	S
	9	73	10	D 68
	10	16	20	S
	11	48	37.5	"
	12	55	50	"
	13	25	100	"
	14	20	100	"
D	1	789	None	D 60
	2	345	"	" 51
	3	210	"	" 86
	4	140	"	" 86
	5	630	4	" 150
	6	345	5	" 105
	7	315	5	" 120
	8	580	6.6	" 72
	9	103	10	" 88
	10	255	20	S
	11	265	26.6	"
	12	180	37.5	"
	13	457	100	"
	14	165	100	"
	15	157	100	"
E	1	5,040	None	D 42
	2	2,400	"	" 77
	3	3,760	4	" 102
	4	7,840	13.3	" 112
	5	5,080	17.5	" 114
	6	8,120	18	" 58
	7	6,720	20	" 60
	8	1,760	50	S
F	1	8	None	D 39
	2	8	"	" 40
	3	8	"	" 38
	4	8	1	" 40
	5	25,440	1.25	" 40

TABLE I—*Concluded*

Determined amounts of enzyme preparations injected intravenously into rabbits 24 hrs. after infective inoculation with Type III *Pneumococcus*

Group	Rabbit	Bacteriemia at 24 hrs.	Units of enzyme	Result
F— <i>concluded</i>	6	$\infty$	5	D 66
	7	20,880	17.5	" 140
	8	13,440	17.5	" 88
	9	$\infty$	20	" 70
	10	$\infty$	20	" 80
	11	$\infty$	20	" 60
	12	37,120	100	" 30
	13	35,840	100	" 36
	14	26,580	100	" 26

S = survival of animal. D = death of animal, at indicated number of hours after infective inoculation.  $\infty$  = number too great to estimate.

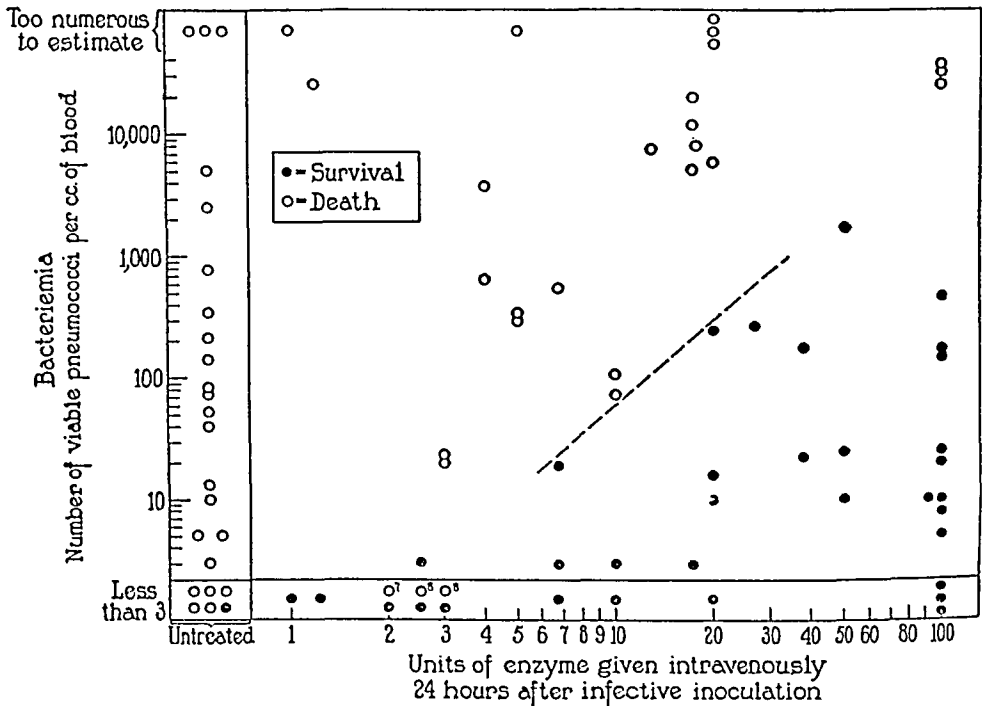
*Group D.*—The animals in this group showed 100 to 1,000 organisms per cc. of blood. In this group all animals which received 20 or more units of enzyme recovered. The controls died somewhat earlier than those of the preceding groups but again the average length of life of the treated animals which died was a day and a half longer than that of the controls.

*Group E.*—In this group are listed the animals with blood cultures showing 1,000 to 10,000 pneumococci per cc. The degree of bacteriemia in this group was very high. In spite of this the animal which received the largest amount of enzyme, 50 units, recovered. Moreover, the animals treated with smaller amounts, although they did not recover, nevertheless lived somewhat longer than did the controls.

*Group F.*—In these animals there were present 10,000 or more pneumococci per cc. of blood. The administration of the enzyme, even as much as 100 units, was without favorable result in these cases. Although in this series of animals receiving but a single injection none recovered, infections of this order have been successfully treated by repeated injections of large amounts of enzyme over a period of days.

Considering Table I as a whole it becomes obvious that larger amounts of enzyme are necessary as the number of organisms in the blood becomes greater. Thus 6.6 units of enzyme were sufficient to bring about recovery in an animal showing 15 organisms per cc. of blood, 20 units sufficed in an animal having 255 organisms per cc., 50 units were required in an animal with 1,760 organisms per cc.; however, a single injection of even 100 units failed to bring about recovery when the number of bacteria per cc. of blood exceeded 20,000.

These results are best illustrated by a graphic presentation as in Text-fig. 1. It will be noted that in general the symbols indicating the fatalities occupy one section of the figure whereas those which indicate the survivals occupy another. The data are not sufficient to allow of the actual plotting of a curve which might separate these areas but a broken line has been drawn which indicates roughly the two zones. The results are not sharply defined in that portion of the chart repre-



TEXT-FIG. 1. Results of the administration of various amounts of specific enzyme in infected rabbits having varying numbers of Type III pneumococci per cc. of blood. A broken line indicates a suggested boundary between zones of effective and non-effective therapy. In three instances the day of death is indicated by small numerals.

senting the animals in which the blood cultures were negative and the amounts of enzyme employed were very small. It is believed that this is due to the fact that in these animals the results depend entirely on the number of organisms found in the lesion and this may of course vary in individual animals.

It may be pointed out that, as the number of pneumococci in the

blood increases, the increase in the relative amount of enzyme necessary to combat the infection becomes less.

#### DISCUSSION

This study has shown that the amount of enzyme necessary to bring about recovery in the infected rabbit bears a definite relationship to the number of organisms present in the blood stream at the time of treatment. Thus, in animals with negative blood cultures or with bacteriemia of low grade, an amount of enzyme as small as 5 units may save the life of the animal; with a bacteriemia of 100 to 1,000 organisms per cc. of blood, 20 units may be necessary; with a bacteriemia of 1,000 to 10,000 organisms per cc., 50 units were necessary. In animals in which the bacteriemia exceeded 10,000 organisms per cc. a single injection of even 100 units was not effectual in saving the life of the animal, although, as previously mentioned, infections of this order have been successfully treated by repeated injections of large amounts of enzyme over a period of days.

Although the quantitative relationship established in these experiments is between the amount of enzyme and the number of pneumococci present in the blood, the fundamental relationship is that between the quantity of enzyme and the total amount of specific capsular polysaccharide present in the body. The degree of bacteriemia serves merely as an index of the latter.

The enzyme is not a therapeutic substance *per se*, but one which, by decomposing the capsular substance of the pneumococci and thus preparing the bacterial cells for phagocytosis, initiates a process which the body must be in a condition to carry on if the animal is to recover. Hence, in the use of the enzyme, this capacity of the body to complete the reaction must be reckoned with.

#### SUMMARY

The enzyme which specifically decomposes the capsular polysaccharide of Type III *Pneumococcus* must be used in certain definite amounts in order to bring about the recovery of rabbits infected intradermally with this organism. The experiments reported in this paper indicate that the minimal amounts of enzyme required bear a definite relationship to the severity of the infection as gauged by the number of pneumococci present in the circulating blood.

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# A STUDY OF THE THERAPEUTIC MECHANISM OF ANTI-PNEUMOCOCCIC SERUM ON THE EXPERIMENTAL DERMAL PNEUMOCOCCUS INFECTION IN RABBITS

## I. THE PRESENCE IN ANTIPNEUMOCOCCIC SERUM OF A NON-ANTIBACTERIAL THERAPEUTIC FACTOR\*

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Despite the numerous clinical and experimental investigations on antipneumococcic serum, its exact rôle in pneumococcus infection is still relatively obscure. The purpose of this communication is to report experiments which show that antipneumococcic serum contains an important therapeutic factor, which is not antibacterial in character.

The current opinion is that the protective antibody in the serum is antibacterial. In 1915, Bull (1), in studying the effect of serum on pneumococcus septicemia in rabbits, suggested that the protective antibody acts *in vivo* by virtue of its agglutinating power, and that the survival or death of the animal depends upon the destruction of the agglutinated organisms by the phagocytic cells of the blood and tissues. Cole (2), Dochez and Avery (3), and others showed that in pneumococcus-infected exudates and sera as well as during the growth of the organisms *in vitro*, there is present a soluble substance, which can combine with the immune bodies of the serum, and thus detract from its action against the organisms. The later studies of Heidelberger and Avery (4), and others on the chemical constitution of pneumococcus protoplasm had established this soluble specific substance as the chief capsular constituent, a carbohydrate fraction of the organism responsible for its type specificity and virulence. The subsequent work of Felton and Bailey (5) indicated that this purified carbohydrate soluble specific substance was capable of neutralizing the protective antibody. Thus it appeared that the func-

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\* I am indebted to Professor William H. Park for his continued interest and stimulating criticism throughout the course of this work. I also wish to thank Mr. M. Percoco, Mr. H. Ferris, Miss A. Lewis, and Miss B. Goldfarb for their technical assistance.

## ACTION OF ANTIPNEUMOCOCCIC SERUM. I

tion of antipneumococcic serum is to combine with the soluble specific substance present either free in the circulation or on the intact organism. This concept of the mechanism, however, fails to explain all the known facts.

For instance, further investigations (6) showed that in addition to the antibody neutralizable by the soluble specific substance (SSS), antipneumococcic serum contains type-specific antibody which is not thus neutralized. This fact was established by demonstrating first, that a certain amount of type-specific protective antibody remains in antipneumococcic serum after complete precipitation of the anti-SSS precipitins, and second, that this residual type-specific protective antibody is neutralized neither by additional SSS nor by absorption with heterologous pneumococci; it is, however, definitely absorbed with the homologous pneumococci. Recently, Ward (7) questioned the validity of this assumption first because he felt that it appeared improbable that the protective action of the serum should be due to several antibodies, and secondly because he was able to show that a Type III serum apparently completely precipitated with Type III SSS, still had a certain amount of antibody, which must have escaped precipitation, and was capable of specifically neutralizing the *in vitro*, specific antiphagocytic effect of small amounts of SSS. With regard to the first contention, it is not at all improbable that the antibacterial effect of a serum may depend on several antibodies reacting with different antigenic components of the organism. Actually the work of Enders (8), and more recently that of Wadsworth and Brown (9) indicate that a type-specific substance other than the so called SSS does exist in coccus Type III is still unknown and, therefore, the comparison is not warranted. With reference to Ward's repetition of the experiments, referred to above (6), it must be stated that he failed to add more SSS to the supernatant liquid of his apparently completely precipitated serum, before testing its neutralization of the antiphagocytic effect of small amounts of SSS. What the results would have been then is, of course, impossible to predict, although theoretically, the solutions, in which the residual type-specific mouse protective antibody was demonstrated (6), because they contained a large excess of SSS would rather be expected to be antiphagocytic *in vitro*. Nevertheless, it is interesting to note that Ward's other experiments suggested that the anti-SSS action is not the only function of the serum.

However, whether there be one, two, or more type-specific antibodies, their action is nevertheless chiefly antibacterial, since they can be specifically absorbed with the intact organisms, and their *in vivo* rôle may still come under the agglutinin effect as conceived by Bull (1). Yet the nature of the factors causing the toxemia and death of pneumococcus-infected animals remains obscure. Certainly the mere presence of the organisms as innumerable microscopic particles cannot account for this effect. Theoretically, therefore, it has been assumed for a long time that the breakdown of the organisms *in vivo* gives rise to toxic substances. The soluble specific substance which neutralizes some of the antibacterial antibody

is apparently *per se* non-toxic to mice, rabbits, or human beings.<sup>1</sup> The studies of Rosenow (10), Cole (11), and others on pneumococcus autolysates showed that while these produce anaphylactic-like reactions in animals, they could not be correlated with the signs of toxemia in pneumococcus infection. In the past few years, Parker (12) has described certain toxic substances obtained by anaerobic autolysis of concentrated pneumococcus suspensions. The toxic principles in these autolysates are capable of producing necrosis when injected intracutaneously and death associated with hemorrhagic pulmonary lesions when injected intratracheally into guinea pigs. These toxins are species-specific and can be neutralized by heterologous anti-autolysate serum. Parker and McCoy (13) reported the production of potent antitoxic serum in horses. However, it appeared that if this toxin prepared *in vitro* by Parker played any part in the *in vivo* process, that the antitoxic serum should act not only on a lesion produced by the artificial toxin, but should exert a definite effect on the course or outcome of the natural infection as well. This problem was investigated using the dermal pneumococcic lesion of rabbits reported by Zinsser (14) and later studied in detail by Goodner (15), as it seemed to be the most suitable, available type of experimental pneumococcus infection, particularly since its course and pathology (Rhoads and Goodner (16)) resemble so greatly that of pneumococcus pneumonia in man. It appeared from this study (17) that the antitoxic serum of Parker, when used by itself or in association with a so called subeffective dose of antibacterial serum, had no apparent effect on the progress of the pneumococcus infection in rabbits. For this reason it would seem that the anaerobically isolated toxin is probably merely a product of *in vitro* autolysis and not actually formed in the course of pneumococcus infection.

The late Dr. E. J. Banzhaf, similarly interested in producing a serum that might perhaps be antitoxic as well as antibacterial, injected horses intramuscularly with pneumococcus broth culture filtrates as well as with the sedimented organisms intravenously. He assumed that in case pneumococcus broth cultures should contain a toxin, which, however, is not readily demonstrable on account of a probable rapid transformation into a toxoid stage, it might, nevertheless, be possible to prepare an antitoxin if they were used for immunization. Banzhaf's preliminary laboratory tests with sera thus produced indicated that some rabbits could be protected with quantities, in which the number of mouse protective units was surprisingly low. He subsequently very kindly submitted to me several samples of this serum for further study, the results of which are included in this report.

The following experiments deal with the demonstration of the presence or absence of therapeutic agents in antipneumococcic serum which:

<sup>1</sup> The author injected four volunteers intramuscularly with as much as 5 mg. of Type I SSS (the equivalent of 200 to 500 cc. of culture) without any harmful effects in three; one man, who had demonstrable agglutinins for Type I pneumococcus in his blood, and was probably hypersensitive, had a rise of temperature to 102.5°F. for 18 hours and marked local pain for 3 days.



are not antibacterial in character. For this study, the dermal pneumococcic lesion of rabbits appeared to be the most suitable, available experimental pneumococcus infection.

#### EXPERIMENTAL

*Pneumococcus Culture.*—A 5 per cent blood broth culture of a fully virulent (*i.e.* one to five diplococci, as determined by colony count in poured plates, killed mice in 3 days or less) Type I *Pneumococcus* was used in all the experiments. The culture was maintained in this state of virulence and supplied for each experiment by Miss Georgia Cooper of the New York City Department of Health Research Laboratories, to whom I am greatly indebted.

*The Dermal Pneumococcic Lesion in the Rabbit.*—Adult rabbits weighing 1500 to 2000 gm. were used. The abdominal hair was removed with electric clippers the day before injection. 1 cc. of an 18 hour blood broth culture was diluted with 1 or 2 cc. of broth to match a turbidity standard, so as to yield approximately 200,000,000 diplococci per cc. The further dilutions were also made with broth, using a fresh pipette for each dilution. 0.1 cc. of a 1-100 dilution of the standardized culture was injected intracutaneously into each rabbit. The number of organisms injected were checked each time by a colony count in poured blood plates.

Rectal temperatures were taken before the injection and daily thereafter throughout the duration of the experiment. Blood cultures from the marginal ear vein were taken daily from each rabbit. The lesions were measured every day noting the size, edema, erythema, hemorrhage, and necrosis. Lesion cultures were not done routinely because only a positive culture was considered significant, and because the elimination of the necessary manipulation was deemed more important as regards these experiments than the additional data that might have been obtained. All rabbits were thus observed for at least 10 days. Each dead rabbit was autopsied and cultures were taken from the heart's blood and from the brain.

#### *Comparison of the Therapeutic Effect of Standard Serum and That Prepared by Intravenous Injection of Organisms and Intramuscular Injection of Broth Culture Filtrate*

The purpose of this experiment was to determine, in a preliminary way, whether or not the antiserum prepared by immunization with organisms and broth culture filtrates was therapeutically more effective in rabbits than the standard serum, prepared by intravenous injections of sedimented organisms only.

In this, as well as in the subsequent experiments, the serum was administered intravenously 6 to 7 hours after the intracutaneous injection of the culture

rather than at 24 hours, as Goodner had done in his studies, because it appeared more suitable for comparative purposes. The virulence of the culture was such that, with the dose used, of forty untreated rabbits in various experiments, none survived; furthermore at 24 hours the disease is frequently very far advanced, some of the rabbits dying within 48 or 72 hours; at 6 hours, however, the lesion is very slight and the blood culture is positive, so that the effect of the serum can be noted not only on the outcome of the disease but on the progress and development of the lesion as well. It is important to note here that protective doses administered at 6 hours do not necessarily influence the full development of the lesion.

TABLE I

*Therapeutic Effect of Standard Antipneumococcic Serum and Serum Prepared by Injections of Organisms and Broth Culture Filtrates*

Therapy	Dose		Hrs. after culture	No. of rabbits used	No. survived
	cc.	m.p.u.*			
None	—	—	—	4	0
Standard Serum 8 Type I	0.38	300	7	4	1
Serum 2321 Type I	5.0	300	7	2	2
	5.0	300	24	2	2
	0.5	30	7	1	0
	0.5	30	24	1	1

\* *M.p.u.*, mouse protective units. The mouse protective unit is ten times the smallest amount of antiserum, which protects a majority of mice for 96 hours, when it is injected intraperitoneally simultaneously with 100,000 fatal doses of a fully virulent culture.

From previous experiments it was known that 300 mouse protective units (m.p.u.) of standard Serum 8 were insufficient for the protection of a majority of rabbits receiving this dose at 6 hours after infection. Serum 2321, produced by intravenous injection of organisms and intramuscular injection of broth culture filtrate, was obtained from a horse that had been under immunization for a relatively short time and its mouse protective potency was rather low. In this experiment four rabbits were untreated; four received 300 units of Serum 8 at 7 hours; 2 rabbits, 300 units of Serum 2321 at 7 hours, and 2 rabbits the same dose at 24 hours; one rabbit, 30 units of Serum 2321 at 7 hours, and one the same dose at 24 hours.

The results are presented in Table I, and show that all the rabbits treated with 300 m.p.u. of Serum 2321 survived, whereas of four rab-

bits treated with 300 m.p.u. of Serum 8, three died. It thus appeared that an equal or similar dose (as regards mouse protective units—the unit which is used clinically as the criterion of dosage) of two different sera was in one instance fully protective for rabbits and in the other practically ineffective. In addition to the variations in the immunization procedures that were used in the preparation of the two sera, there was still another difference: standard Serum 8 contained the 300 m.p.u. in 0.38 cc., whereas Serum 2321 contained the same number of units in 5 cc. The question was, therefore, whether the greater effectiveness of Serum 2321 was due to the added intramuscular injections of broth culture filtrate employed in its preparation, or to a factor present in both Serum 2321 and Serum 8, more of which was associated with the 300 m.p.u. of the former on account of the larger volume of the dose. In either case, however, this factor would have to be of a nature different from the agent which is determined by the present method of mouse protection. By absorbing an antipneumococcic serum with the homologous organism it is possible to remove almost all (about 99.5 per cent) of its mouse protective capacity; it was, therefore, important to determine whether the supernatant liquid of a serum thus absorbed could exert any therapeutic effect in rabbits.

*The Non-Antibacterial Therapeutic Factor in Serum Prepared by Injection of Organisms Intravenously and Broth Culture Filtrate Intramuscularly*

In the work done on the rôle of anaerobic toxins in the dermal pneumococcic lesion of the rabbit (17), as well as in the previous experiment it was apparent that for every serum there is a certain subeffective dose. Of a group of rabbits treated with this subeffective dose there is at first a sterilization of the blood stream in all; in less than half the blood is reinfected whereas in the others it remains sterile; the dermal lesion is not markedly influenced; about 60 to 75 per cent die. Approximately half of these rabbits die without reinfection of the blood stream and with a sterile postmortem heart's blood culture, but usually with a progressively marked lesion. (Routine postmortem brain cultures, which were done on all the rabbits reported in these experiments, showed that, in the absence of a continued and persistent bacteremia, localization of the infection in the central nervous system was rela-

tively extremely rare.) It appeared, therefore, that in these cases death is produced by the absorption of toxins from the local lesion.

Thus, it was essential to determine whether a subeffective dose of serum could be rendered effective by the addition of that fraction of the serum which remains after complete absorption with the homologous organisms.

TABLE II

*The Non-Antibacterial Therapeutic Factor in Serum Prepared by Injection of Organisms and Broth Culture Filtrates*

Therapy	Dose		Hrs. after culture	No. of rabbits used	No. survived
	cc.	m.p.u.			
Preparation 4	0.2	50-100	6	9	4
Preparation 4 +	0.2	50-100	6	3	1
Absorbed Preparation 4	0.4	0.4-2.0	24		
Preparation 4	0.4	100-200	6	5	2
Preparation 4 +	0.4	100-200	6	5	5
Absorbed Preparation 4	0.6	0.6-3.0	24		
Preparation 4 +	0.4	100-200	6	5	5
Absorbed Preparation 4	0.25	0.25-1.25	24		
	0.25	0.25-1.25	48		
	0.25	0.25-1.25	72		
Standard Serum 624 Type I	0.3	300	6	4	1
Serum 624 +	0.3	300	6	4	4
Absorbed Preparation 4	0.5	0.5-2.5	6		
	0.5	0.5-2.5	24		

The serum available for this experiment was a concentrated preparation (Preparation 4) containing the total water-soluble and water-insoluble globulin (precipitated at 30 to 50 per cent saturation with  $(\text{NH}_4)_2\text{SO}_4$ ) of Serum 2321 used in the previous experiment. This preparation contained 250 to 500 m.p.u. per cc. Preparation 4 was absorbed with heat-killed, sedimented pneumococci until no further agglutination occurred; when this stage was reached more organisms were

added, and the mixture incubated at 37°C. for 2 hours and kept in the refrigerator overnight. After sedimenting the unagglutinated organisms by thorough centrifugation, the supernatant liquid was poured off and called absorbed Preparation 4. A mouse protection test<sup>2</sup> performed with absorbed Preparation 4 showed that it contained 1 to 5 units, indicating an absorption of about 99.5 per cent of the original mouse protective capacity. In a preliminary experiment a subeffective dose of Preparation 4 was determined, then one group of rabbits was injected with a subeffective dose of Preparation 4 at 6 hours, and other groups received, in addition to the subeffective dose at 6 hours, injections of absorbed Preparation 4 at 24 hours, 48 hours, and 72 hours as shown in Table II.

The survival rate of the rabbits indicates that 0.2 and 0.4 cc. of Preparation 4 constitute subeffective doses. It is also clearly apparent that the additional injection of 0.6 cc. of absorbed Preparation 4 at 24 hours or of 0.75 cc. divided in three equal doses at 24 hour intervals rendered the subeffective dose therapeutically effective, all the rabbits thus treated having survived. As confirmatory evidence, it was necessary to determine whether absorbed Preparation 4 would have a similar effect when added to a subeffective dose of standard antibacterial serum; *i.e.*, one prepared by immunization with organisms only. The results shown in Table II again indicate that Preparation 4, after complete absorption with the homologous organisms, contains a substance which exerts a definite therapeutic effect on pneumococcus infection in rabbits. For convenience, this therapeutic factor was called non-antibacterial to indicate merely that it was not absorbed or neutralized by the intact homologous organisms, and not to define its mode of action. If this non-antibacterial factor is of the nature of an antibody or antitoxin, it apparently must be produced in response to some soluble toxic substance, which, however, is not an antigenic constituent of the intact organism.

#### *Can Pneumococcus Broth Culture Filtrate Neutralize the Non-Antibacterial Factor?*

Since broth culture filtrate was the additional immunizing agent used in the preparation of the serum, which was shown to contain the non-antibacterial therapeutic factor, it was important to determine

<sup>2</sup> The mouse protection tests reported in these experiments were performed in the laboratory of Miss Georgia Cooper of the New York City Health Department Research Laboratories, to whom the author again wishes to express his gratitude.

whether or not it would be capable of neutralizing this factor. It seemed that no causal relationship between the use of broth culture filtrate for immunization and the presence of the non-antibacterial factor in the serum could be established, unless such neutralization were demonstrable.

1000 cc. of a 24 hour *Pneumococcus* Type I broth culture were passed through a large Seitz filter. The filter was then concentrated by a method commonly used

TABLE III  
*Effect of Broth Culture Filtrate on Non-Antibacterial Factor*

Therapy	Rabbit No.	Result	Remarks
0.3 cc. Serum 624 (300 units) + 1.5 cc. absorbed Preparation 4	1	S	Marked lesion; bacteremia controlled; postmortem heart and brain cultures sterile
	2	S	
	3	S	
	4	D <sub>6</sub> *	
0.3 cc. Serum 624 (300 units) + Mixture containing 1.5 cc. absorbed Preparation 4 and 3.0 cc. alcoholic pneumococcus broth concentrate	5	S	Bacteremia uncontrolled
	6	D <sub>4</sub>	
	7	S	
	8	S	
0.3 cc. Serum 624 (300 units) only	9	S	Marked lesion; postmortem heart and brain cultures sterile
	10	D <sub>4</sub>	
	11	D <sub>4</sub>	
	12	D <sub>9</sub>	
	13	D <sub>8</sub>	

\* S = survived. D<sub>6</sub> = died 6 days after injection.

in obtaining diphtheria toxin from broth cultures. The filtrate was acidified with glacial acetic acid and precipitated with 60 per cent alcohol; after remaining in the refrigerator overnight, the precipitate was filtered through paper. It was then pressed nearly dry between pieces of filter paper and dissolved in 50 cc. of 0.9 per cent NaCl; i.e., in 1/20 the original volume. 2 cc. of this concentrate injected intravenously into two normal rabbits produced no symptoms. To one part of Preparation 4, which previously had been completely absorbed with heat-killed,

washed Type I pneumococci, two parts of the pneumococcus broth concentrate were added. The mixture was incubated in the water bath at 37°C. for 2 hours and kept in the refrigerator overnight. A small amount of precipitate had formed; this was centrifuged and the clear supernatant liquid was used for injection into rabbits. Four rabbits received a subeffective dose of standard Serum 624 together with 1.5 cc. of absorbed Preparation 4; four other rabbits—Serum 624 and a mixture of 1.5 cc. absorbed Preparation 4 treated with 3 cc. of the pneumococcus broth concentrate; five rabbits were injected with the subeffective dose of Serum 624 only, for control. All serum was given intravenously 6 hours after the intracutaneous injection of the organisms.

The results shown in Table III indicate that the concentrated pneumococcus broth culture filtrate failed to neutralize the therapeutic effect of absorbed Preparation 4. It thus appeared that if the broth culture filtrate does not neutralize the non-antibacterial therapeutic factor, it probably also does not contain its antigen, and that, therefore, serum prepared by the standard method of intravenous injection of organisms only, should similarly contain this factor.

*Presence of the Non-Antibacterial Therapeutic Factor in Serum Prepared by the Intravenous Injection of Organisms Only*

The purpose of this experiment was to determine first, whether or not the non-antibacterial therapeutic factor can be demonstrated in serum prepared by the intravenous injection of organisms only, and second, whether it is type-, species-, or non-specific. For control, normal horse serum, and Parker's so called antipneumotoxin, prepared by immunizing horses with the toxic, anaerobic, pneumococcus autolysate, were used.

The Type I sera were absorbed with Type I, heat-killed, washed pneumococcus suspensions; the heterologous serum, normal horse serum, and Parker's antipneumotoxin were not absorbed with any organisms, because a test showed that they contained less than 1 mouse protective unit per cc. for Type I pneumococcus. Four Type I monovalent sera, one Type II monovalent serum, and one normal horse serum are included in the tabulation of the results. The tests were carried out at different times on several different groups of rabbits. To determine whether the non-antibacterial factor could be demonstrated in a serum, other than antipneumococcic, one group of rabbits was tested with a scarlatinal, antibacterial, and antitoxic serum obtained from a horse that at no time had received any injections of pneumococci. The dose varied from 2 to 5 cc. for each serum tested. The results of all the tests are summarized in Table IV.

Of the forty rabbits that were untreated in the various experiments none survived. The striking contrast between the survival rate (29.0 per cent) of those receiving the subeffective dose of Type I serum only with that (81.4 per cent) of the rabbits receiving the additional injection of pneumococcus-absorbed Type I serum, indicates very definitely the presence of a similar non-antibacterial, therapeutic factor in serum prepared by the intravenous injection of organisms only. The results with the Type II serum are almost equally striking, and suggest that this non-antibacterial factor is not type-specific. The apparent absence of the factor in the antipneumotoxin is of

TABLE IV

*The Non-Antibacterial Therapeutic Factor in Standard Antipneumococcic Serum*

Serum therapy	No. of rabbits used	No. survived	Survival
			<i>per cent</i>
None.....	40	0	0
Subeffective dose of Type I antiserum.....	31	9	29.0
Subeffective dose of Type I antiserum + absorbed Type I serum.....	43	35	81.4
Subeffective dose of Type I serum + heterol- ogous serum Type II.....	17	12	70.6
Subeffective dose of Type I serum + Parker's antipneumotoxin .....	22	6	27.3
Subeffective dose of Type I serum + normal horse serum.....	10	4	40.0
Subeffective dose of Type I serum + scarla- tinal antibacterial and antitoxic serum....	10	3	30.0

interest with regard to its mode of origin. The survival rates of the rabbits which received the normal horse serum and the scarlatinal, antibacterial and antitoxic serum, considering the smaller number of animals, are well within the range of survival of those treated with the subeffective dose only, the results indicating that this therapeutic factor probably is not non-specific. Considering the fact that standard antipneumococcic sera contain, in addition to the antibacterial antibody, other therapeutic factors which apparently are non-antibacterial, one can understand more readily how equal doses, as regards mouse protective unit content, may vary in therapeutic efficiency.



TABLE V  
*Therapeutic Effect of Non-Antibacterial Factor per Se and in Conjunction with a Subeffective Dose of Serum*

Therapy	Experiment No.	Rabbit No.	Dermal lesion	Bacteremia	Result	Postmortem cultures	
						Heart's blood colonies per cc.	Brain
Absorbed Preparation 4, dose 2 cc. contained 2-10 m.p.u.	(a)	1	Very slight	Controlled	S	1000	Sterile
		2	"	Uncontrolled	D <sub>4</sub>		
		3	Slight	Controlled	S		
		4	"	"	S		
	(b)	5	Very slight	Controlled	D <sub>2</sub> * D <sub>7</sub>	Sterile "	" "
		6	Marked	"	D <sub>11</sub>	"	"
		7	Moderate	Marked; uncontrolled	D <sub>2</sub>	Innumerable	Positive
		8	None	Controlled	S		
	(c)	9	Slight				
		10	Very slight	Controlled	S	Innumerable	Innumerable
		11	"	Uncontrolled	D <sub>4</sub>		
		12	"	Controlled	S	Innumerable	Positive
		13	Moderate	Uncontrolled	D <sub>7</sub>		
		14	"	Controlled	S	Innumerable	Sterile
		15	None	Uncontrolled	D <sub>3</sub> †		
		16	Marked	Controlled	S		
Absorbed Serum 348, dose 0.75 cc. contained about 4 m.p.u.	(c)	17	Marked	Controlled	S	Sterile	Sterile
		18	"	"	D <sub>7</sub>	500	"
		19	Very marked	" 2 days	D <sub>3</sub>	Sterile	"
		20	"	"	D <sub>3</sub>	Innumerable	Innumerable
		21	"	" 4 days	D <sub>6</sub>		

Absorbed Serum 348, 0.2 cc.—1 m.p.u. + Serum 624, Type I, 0.3 cc.—300 m.p.u.‡	(c)	22	Very marked	Controlled " 2 days " " " "	S D <sub>7</sub> † D <sub>10</sub> S D <sub>3</sub>	Innumerable Sterile Sterile	Positive Sterile Sterile
		23	Slight				
		24	Very marked				
		25	Moderate				
		26	Marked				
Absorbed Serum 348, 0.5 cc.—2.5 m.p.u. + Serum 624, Type I, 0.3 cc.—300 m.p.u.	(c)	27	Slight	Controlled Uncontrolled Controlled " "	S D <sub>3</sub> S S D <sub>11</sub>	Innumerable Sterile	Positive Sterile
		28	Marked				
		29	Very slight				
		30	Slight				
		31	Very marked				
Absorbed Serum 348, 1.0 cc.—5 m.p.u. + Serum 624, Type I, 0.3 cc.—300 m.p.u.	(c)	32	Slight	Controlled " " " "	S S S S S		
		33	Marked				
		34	Very marked				
		35	Marked				
		36	Very slight				

S = survived; D<sub>1</sub> = dead 4 days after infection. *M.p.u.* = mouse protective units.

\* With a negative lesion, absent bacteremia, and entirely negative postmortem findings, the cause of death is not clear.

† With no gross dermal lesion, there were post mortem a hemorrhagic infiltration of both lungs and an acute fibrinous pericarditis.

‡ Hemorrhagic infiltration of both lungs; acute pleuritis and acute pericarditis.

§ See Tables II, III, and IV for effects of subeffective dose of Serum 624 when administered by itself.

*Effect of the Non-Antibacterial Therapeutic Factor per Se*

It was essential to determine the effect, if any, of a pneumococcus-absorbed serum, that is known to contain the non-antibacterial factor, when injected by itself without the addition of a certain subeffective dose of antibacterial serum. This experiment was expected to yield information regarding the rôle of the non-antibacterial factor as well as of the mouse protective antibody which is absorbable by the intact organism.

Two preparations were tested: (a) absorbed Preparation 4 which has been described previously, and (b) absorbed Serum 348, which was an unconcentrated monovalent Type I serum of at least 2000 m.p.u. per cc., and which after absorption with the homologous pneumococcus still contained about 5 m.p.u. per cc. The dose of absorbed Preparation 4 for each rabbit was 2 cc.; the dose of absorbed Serum 348, 0.75 cc. Three groups of rabbits were tested on different occasions; the results are shown in Table V.

It is necessary to recall that both Preparation 4, which is a concentrated (about 5 times) globulin preparation, and Serum 348 were so treated that the final absorbed preparations contained no *in vitro* demonstrable agglutinins, which involves a neutralization of the immune bodies not only by the SSS but by any other antigen that may be present on the intact organism. Whether or not the small amount of mouse protective antibody which is still demonstrable in these absorbed preparations is of an antibacterial or non-antibacterial nature cannot be definitely stated as yet, for it is not improbable that during the absorption of antibody an equilibrium may be reached wherein a certain small amount of antibody remains uncombined. Similarly it must be stated that the absorbed preparations probably do not contain the total quantity of non-antibacterial factor that is present in the serum since some of it may become non-specifically adsorbed during the precipitation of antibody on the bacteria.

The results presented in Table V show most interestingly, particularly in view of the fact that untreated rabbits do not survive with this dose of pneumococcus (see Table IV), that a certain number of rabbits is protected by the completely absorbed serum; eight of the sixteen rabbits treated with absorbed Preparation 4 and one of the five treated with absorbed Serum 348 were fully protected. A simul-

taneous titration of absorbed Serum 348 administered with a standard subeffective dose of Serum 624, indicates that approximately 0.5 to 1.0 cc. is efficient for rendering it effective. The difference in survival rate between the absorbed Preparation 4 and absorbed Serum 348 groups appears to be a matter of dosage more as regards the non-antibacterial factor than the antibacterial antibody, for the rabbits that died in the former group had a terminal septicemia, whereas two of the rabbits (Nos. 18 and 20) in the latter group died without any bacteremia but apparently by absorption of toxin from the marked focal lesions. Furthermore, it is important to note that in fourteen of the first twenty-one rabbits, the bacteremia was completely controlled. Whatever the nature of the residual mouse protective antibody, the amount, contained in the doses that were used, is relatively so very small that one is led to think that either only a very minute quantity of the antibacterial antibody is required for the control of bacteremia, or else that it plays a secondary rôle particularly when septicemia becomes very marked. In the last three groups of rabbits (Nos. 22 to 36), one observes how with a relatively standard mouse protective unit dose, the bacteremia is more readily controlled as the dose of the non-antibacterial factor is increased. With the larger dose of the non-antibacterial factor, as in absorbed Preparation 4, most of the rabbits showed very mild dermal lesions as compared not only with control, untreated rabbits but also with those which received an insufficient dose. When one contrasts with this the action of a standard subeffective dose that contains a relatively large amount of antibacterial antibody, in which case the dermal lesions are marked and death occurs frequently without bacteremia, the suggestion is very strong that the ultimate outcome of the infection depends to a great extent upon the non-antibacterial therapeutic factor. The effect of the non-antibacterial factor suggests that it opposes, in some way as yet unknown, the action of the products of infection which locally are responsible for a marked dermal lesion, and systemically are probably the cause of death.

#### DISCUSSION

The work reported in this communication is in line with an attempt to determine the mechanism of death and recovery in pneumococcus

infection. The rôle of the antibacterial antibodies, which are capable of combining with the various constituents of the bacterial protoplasm, is ultimately, as was pointed out by Bull, to enhance phagocytosis of the organisms by cells of the blood and tissues. With the thought that the mere multiplication of organisms as so many microscopic particles cannot logically be the cause of death, the view that phagocytosis is the chief factor which determines death or recovery appears to be unsatisfactory in many respects. The constituents which are isolated from virulent pneumococci *in vitro* and against which antibodies are demonstrable in the standard antipneumococcic sera, are non-toxic; yet when the intact, living organisms are introduced into the body of a susceptible animal, a most potent toxin is apparently elaborated. The question arises, therefore, whether antipneumococcic serum is effective only when through preventing the multiplication of the organisms it also prevents the formation of a lethal amount of toxin, or whether it possesses an action against this toxin as well.

Since in the method of mouse protection, which is at present used as the criterion for determining the potency of antipneumococcic sera, the serum is injected simultaneously with a relatively very small number of organisms into the same body cavity, the function which is primarily involved is that which brings about their phagocytosis, and prevents the multiplication of organisms to the extent that a lethal dose of toxin is not produced. For this reason this method was deemed unsuitable in the present study. The dermal pneumococcus lesion of the rabbit was chosen because it provided a focus where the organisms may be localized and from which, under conditions which are unsuitable for the systemic multiplication of the bacteria, sufficient toxin may be absorbed to cause death of the animal.

The first serum to be investigated was one prepared by the late Dr. E. J. Banzhaf, who immunized several horses not only by the intravenous injection of sedimented pneumococci (which is the routine practice), but also by the additional intramuscular injection of broth culture filtrates. Preliminary experiments showed that when certain similar doses, as regards mouse protective potency, of Banzhaf's serum and one prepared by the standard procedure, were tested on rabbits with the experimental dermal pneumococcus infection, the former was found to be fully protective and the latter practically in-

effective. It was thus apparent that the protective effect did not depend only on the number of mouse protective units and that some factor which was not determined by the mouse protection test, was present in one serum and absent in the other, or else present in both, but in different concentrations. The subsequent experiments were designed to determine whether this factor was of antibacterial or non-antibacterial nature. By absorbing an antipneumococcic serum with a heat-killed suspension of homologous pneumococci it is possible to remove almost all (about 99.5 per cent) of its mouse protective antibody. When this antibody was absorbed from the serum, prepared by immunization with organisms and broth culture filtrates, and the remaining supernatant liquid was administered with a subeffective dose (see Table II) of the original serum, it was found that this combined therapy proved fully effective. It appeared, therefore, that the difference between an effective and ineffective dose was not only the antibacterial antibody (*i.e.* that which is absorbed with the intact organisms) but also another factor which is apparently non-antibacterial. Furthermore, it was shown that a certain dose of such an absorbed preparation was by itself therapeutically effective in about half the treated rabbits. In the same experiment an analysis of the course of the bacteremia in the various rabbits indicated that either only a minute amount of the antibacterial antibody is required for the control of the bacteremia or else that it plays merely a secondary rôle only when the septicemia becomes very marked. These results again pointed to the fact that the ultimate outcome of the infection is dependent to a considerable extent upon the non-antibacterial therapeutic factor.

However, no definite causal relationship could be established between the additional injections of broth culture filtrates for immunization and the presence in the serum of the non-antibacterial factor, because (*a*) a concentrated preparation of broth culture filtrate failed to neutralize it, and (*b*) standard serum, prepared by the intravenous injection of sedimented organisms only, also contained this factor. It was further demonstrated that the non-antibacterial factor is not type-specific, and that it is probably species-specific. If this factor is of the nature of an antibody or an antitoxin, it must necessarily have its corresponding antigen. Such an antigen, by the very definition of the non-antibacterial factor, is not present in the intact or-

ganism; a concentrated broth culture filtrate did not contain it; and judging from the results obtained in these experiments, it is apparently also absent in the *in vitro* anaerobic autolysates of pneumococcus; however, it is not inconceivable that it may originate from the pneumococcus *in vivo*. From this point of view, it is interesting to consider the recently proposed conception of Curphey and Baruch (18). On purely theoretical grounds, they proposed the possibility that the immune bodies developed in the course of lobar pneumonia include antibodies which are formed as a result of the products of tissue cell destruction either *per se* or in combination with certain bacterial products. On the basis of this conception, they have prepared antipneumococcic serum by immunization with pleural exudates from pneumococcus-infected horses. In comparing (19) similar doses, as regards mouse protective units, of their serum with that of standard serum, using the dermal pneumococcus infection in rabbits, they found the former therapeutically more effective; however, since standard sera were shown to contain a non-antibacterial factor, and on account of the fact that the volume of the doses of the two sera was not taken into consideration, one cannot definitely assign the greater therapeutic effectiveness to the mode of immunization. The possibility, nevertheless, remains and deserves further investigation. It is also interesting to correlate the non-antibacterial therapeutic factor with Tillett's (20) observations on the production of immunity in rabbits against virulent strains by injections of R pneumococci; this immunity was not type-specific, and the serum of rabbits thus immunized, although it contained no demonstrable mouse protective antibody, was, nevertheless, capable of protecting normal rabbits against virulent Types I, II, and III pneumococci.

The fact, that antipneumococcic serum was shown to contain a therapeutically active non-antibacterial factor, may have an important bearing on the serum treatment of pneumococcus pneumonia in man, and suggests many new problems some of which have been investigated and will be reported in the next communication.

#### CONCLUSIONS

1. Antipneumococcic serum contains in addition to the antibodies against the various bacterial constituents, a non-antibacterial therapeutic factor.

2. The non-antibacterial factor has been separated from the antibacterial antibodies by absorption with homologous, heat-killed, virulent pneumococci.

3. The non-antibacterial factor is not type-specific; it is probably species-specific; it is not neutralized by concentrated pneumococcus broth culture filtrate.

4. The therapeutic effect of the non-antibacterial factor was demonstrated on the experimental, dermal pneumococcus infection of rabbits; this effect is demonstrable when it is administered *per se* or in conjunction with a certain subeffective dose of serum.

5. Evidence is presented for the assumption that the ultimate outcome of pneumococcus infection depends to a considerable extent upon this non-antibacterial factor.

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THE FORMATION OF MACROCYTES AND MICROCYTES  
FROM RED CORPUSCLES IN HANGING  
DROP PREPARATIONS\*

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INTRODUCTION

While studying the process of contraction in blood coagulating under normal and abnormal conditions, red corpuscles were observed in the microscope field which were markedly larger or markedly smaller than normal. Moreover these peculiar forms appeared in the same preparations which had not shown them previously. As a survey of the literature revealed no similar observations, the formation of these structures was studied. It became necessary to follow the fate of the same corpuscle for hours at times in order to obtain definitive evidence that a normal red corpuscle could change into a macrocytic or a microcytic form. The following descriptions therefore do not represent a mere record of isolated morphological observations linked together by hypothesis, but they represent a more or less extensive series of functional and morphological changes that were directly observed in one specific cell or group of cells during the course of seconds, minutes or hours; examples are given in the outline drawings and their legends which accompany this article.

*Technique*

The blood specimens were studied chiefly in hanging drop preparations without the addition of any diluent or foreign substance. Ordinary micro culture slides with two wells 18 mm. wide and 1.5 mm. deep were used. The cover-slips were the thinnest obtainable. Both slides and cover-slips were cleaned with a Bon Ami

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\* A preliminary note was published in *Proc. Soc. Exp. Biol. and Med.*, 1930, 27, 620.

suspension, washed in distilled water and with 95 per cent undenatured alcohol, then dried with a soft cotton cloth. Before use both were flamed with a Bunsen burner.

The preparation was sealed with petrolatum which had been boiled in water on 3 successive days. The vaseline was applied to the four edges of the cover-slip before taking the blood drop.

All observations were made with a Zeiss apochromat microscope and a 3 mm., 1.4 N.A. oil immersion objective, except in some earlier work where a 2 mm., 1.3 N.A. oil immersion lens was used. The oculars employed were K 10, K 15 and K 20. The magnifications therefore ranged between 600 and 1200; the lower magnification was used most frequently.

Measurements were made with an ocular micrometer (K 7 X) calibrated with a stage micrometer ruled in 0.01 mm. One scale division of the ocular micrometer with the 3 mm. objective and a tube length of 160 mm. equalled  $2.6\mu$ . An error in the estimation of 0.1 of a scale division thus represents  $0.3\mu$ . Though all measurements recorded in this paper therefore may possess an error of  $0.3\mu$ , it was deemed best to record them just as they were noted in the protocols.

A Zeiss filament lamp with Florence flask light filters furnished the light.

The blood was obtained from 10 apparently normal human subjects; 10 dogs; 31 rabbits, and 21 guinea pigs. The present report is based on more than 100 satisfactory preparations and each one was under continuous observation for periods lasting 1 to 6 hours. The slides were generally reexamined after 24 and 48 hours, at times also after 72 and 96 hours. In some favorable instances observations were possible 12 to 14 days after the preparation had been made.

Human blood was obtained by puncturing the congested finger tip with a triangular needle; in the other animals an ear vein was cut transversely. The site was always shaved when necessary and then cleansed with undenatured alcohol. The first drop was wiped off with gauze and the next freely flowing drop of appropriate size was touched to a flamed cover-slip rimmed with vaseline and then inverted over the well of a slide.

All specimens were generally prepared between 9 a.m. and 3 p.m. All observations were made at room temperature.

The hemoglobin content was determined with a Dare hemoglobinometer before taking the sample for microscopic examination.

The red corpuscles of the various species were round or slightly oval discs, light yellowish brown in color, and all exhibited a more or less marked dellen in the beginning of the examination. The transverse diameter varied in the different species and in the species itself. In normal, male human blood, variations between  $5.6$  to  $8.4\mu$  were observed; the ordinary range however was less marked:  $7.8$  to  $8.4\mu$ . In dog the average transverse diameter was  $7.8\mu$ . In rabbit, chiefly males, the extremes noted were  $5.0$  to  $7.8\mu$ , the usual fluctuation being  $6.5$  to  $7.5\mu$ . In the adult male guinea pig the diameter ranged between  $6.0$  to  $8.4\mu$ .<sup>1</sup>

<sup>1</sup> For comparison see Bürker, K., in Bethe, A., von Bergmann, G., Embden,

## OBSERVATIONS

*Definition of Macrocyte and Microcyte.*—In the following pages the term macrocyte means a giant red cell which has been formed by the fusion of two erythrocytes; it has generally the same hemoglobin tint as a normal red corpuscle; its shape is round or slightly oval with a transverse diameter of  $10\mu \pm$ ; there is no dells; thickness measurements could not be made, but the general shape seems to be discoid since most of the visible surface lies in one focal plane.

The term microcyte means a disc-shaped red corpuscle which has been seen forming from an apparently normal red corpuscle; its diameter is  $5\mu \pm$ ; its thickness on rolling over varies between 2.5 to  $3\mu$  (rabbit), and then it appears as a brownish yellow cylinder with rounded ends and parallel sides, though occasionally a slight entasis is observable; there is no dells and the hemoglobin tint is originally always much deeper than that of a normal red corpuscle. No reliable thickness measurements were obtainable on macrocytes or blood shadows.

These microcytes are probably identical with the bodies described by Quincke<sup>2</sup> in pernicious anemia and perhaps also with those noted by Eichhorst<sup>3</sup> in the same disease. In Eichhorst's report, however, the diameter is given as one-quarter that of a normal red corpuscle which would be approximately  $2\mu$ ; Quincke reports their diameter as 4 to  $6\mu$ . In my work the microcytes were astonishingly uniform in diameter measuring approximately  $5\mu$ .

*The Formation of Macrocytes.*—In the following description no distinction is made between the various species of blood because the basic processes were the same in all. It may be emphasized again that the present study describes the progress of changes which were seen in a selected single cell or group of cells. Necessarily a complete series of observations on one cell was obtained in relatively few preparations, but these were invaluable because they could serve as trustworthy guides in interpreting fragmentary evidence.

After a hanging drop preparation is made, immediate examination

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G., and Ellinger, A., *Handbuch der normalen und pathologischen Physiologie*, Berlin, Julius Springer, 1928, 6, 1st half, pt. 1, 15.

<sup>2</sup> Quincke, H., *Deutsch. Arch. klin. Med.*, 1877, 20, 19.

<sup>3</sup> Eichhorst, H., *Centr. med. Wissensch.*, 1876, 14, 466.

of the thin, peripheral plasma serum edge usually discloses lacking red corpuscles and shadows. Careful inspection of this area sooner or later reveals numbers of corpuscle pairs which touch each other to a varying degree; the dells of each is slightly marked or imperceptible. If such a couple is continuously examined it may be seen that the site of contact gradually increases until the two discs form an oval mass encircled at or near its equator by a delicate groove. Careful focussing discloses a whitish line in the middle of this groove and this line represents the apposed or fusing surfaces of contact. These apposed surfaces form a single septum which generally remains perceptible for some time. The septum finally disappears but its presence may still be indicated by slight peripheral indentations of the contour. Later these disappear also and now a round or slightly oval macrocyte is seen whose transverse diameter approaches or exceeds  $10\mu$  and in which no septum can be detected (Fig. 1, Group A). This rounded macrocyte possesses no dells and its entire surface seems to be practically in one focal plane; its hemoglobin tint is apparently identical with that of a normal red corpuscle. None of these fused corpuscles was ever definitely seen in profile, but the evidence given indicates a disc shape.<sup>4</sup>

Fusing corpuscles of this type may be observed within 2 minutes after making the preparation, and the process may be complete in 10 minutes or less. After its formation the macrocyte may show no apparent change for many minutes; within 30 minutes however the structure usually begins to pale. This fading often takes place abruptly and occasionally is initiated by a slight general jerk which does not deform the contour of the macrocyte. Now in less than 1 second the hemoglobin has left the giant corpuscle and only a shadow of the same size and contour remains. This astonishing speed of hemolysis accounts for the sudden disappearance of corpuscles which were lost while the eye was momentarily glancing at some other section of the field. For example a giant corpuscle without septum or dells and with the hemoglobin tint of a normal red corpuscle was carefully

<sup>4</sup> Several times macrocytes were observed slowly changing from a round to an oval shape and then back to the original round contour; possibly this represents a rolling over of the corpuscle; it may, however, have been caused by variations in stroma tension.

observed for 44 minutes. Only a slight paling in color became noticeable and the outline of the cell had exhibited slight, local, transitory bulgings during this period. This macrocyte suddenly disappeared while the eye was inspecting another portion of the field. There were no currents or sudden contractions of the field at the time. The newly formed shadow may exhibit a slight yellow tint or it may be paler than the background.

The process outlined above does not always advance as smoothly as the description perhaps indicates, nor does it always end in hemolysis. The stages intervening between the moment when two red corpuscles touch and the laking of a resultant giant form may vary considerably in duration in different samples of blood from the same species and from different species. The fusion may be completed in 2 minutes; it may consume 25 minutes or more; the fusion may remain incomplete, a median septum being visible up to the time of hemolysis; or finally the two apposed corpuscles may separate after a time without any change in contour or hemoglobin content.

Fusion occurs not only between two apparently normal red corpuscles; it may be observed between a red corpuscle and a microcyte (Fig. 1, Group B) or between two microcytes (Fig. 1, Group C). The course of events is essentially the same in all.

*Washed Corpuscles.*—A number of experiments on the incidence of fusion in washed rabbit and guinea pig corpuscles were made. About 0.5 cc. of blood was shaken with 9 cc. of mammalian Ringer solution and then centrifuged. This was repeated 4 to 6 times and then a hanging drop was prepared. In these slides fusing red corpuscles were noted after 2 to 4 hours, except in one instance where 21 hours elapsed. In this latter instance the fusions were exceptionally plentiful.

*Stagnating Blood.*—Some experiments were also made to study the influence of stasis on the production of macrocytes. The stasis was produced in the rabbit by clamping the lateral ear vein after folding the ear longitudinally; in the human subject the finger was tightly compressed by means of a rubber tube after congestion had set in. The duration of stasis varied between 5 to 10 minutes. Both in rabbit and human blood, fusing corpuscles appeared 5 to 8 minutes after making the hanging drop and were not more numerous than under

normal conditions. In both kinds of blood the transverse diameter of normal corpuscles was diminished: in the rabbit for example, the transverse diameter varied between  $5.2$  and  $6.5\mu$  and the hemoglobin tint of each corpuscle appeared deeper.<sup>5</sup>

*Bone Marrow and Spleen Pulp.*—In a few animals a rib was resected under morphine anesthesia, and a few days later the medulla was pithed and the spleen removed at once. It was necessary to add Ringer solution to the expressed bone marrow and spleen pulp in order to prevent drying during examination. No fusing or fused red corpuscles were seen at all in these preparations of the rabbit and guinea pig. Fibrin threads were practically absent and only a few spindle-shaped corpuscles were observed.

*Formation of Microcytes.*—Four methods have been observed by which microcytes develop from normal blood corpuscles in hanging drop preparations.

1. If the blood clots fairly rapidly, the periphery of a hanging drop shows spindle- or pear-shaped red corpuscles. These changes are caused by the pull of fibrin threads attached to the corpuscle.<sup>6</sup> During observation one of these corpuscles may be abruptly deformed to a lozenge shape; after a pause, with equal abruptness a star-shaped figure appears without any alteration of hemoglobin tint. Suddenly the remaining anchoring fibers snap or relax and the angular structure recoils with vigor into a round form with short, stubby projections at the sites where the fibrin threads pulled. The visible area is definitely smaller than that of the original corpuscle, the hemoglobin tint is deeper and a delle is not detectable. In a few additional seconds the stubs disappear and now a perfect smooth microcyte with a transverse diameter of  $5\mu$  has been formed (Fig. 1, Group D). The whole process may be complete in less than 1 minute.

Complete series of this type were seen not only in the guinea pig

<sup>5</sup> For the literature bearing on variations in corpuscular size see Bürker, K., in Bethe, A., von Bergmann, G., Embden, G., and Ellinger, A., *Handbuch der normalen und pathologischen Physiologie*, Berlin, Julius Springer, 1928, 6, 1st half, pt. 1, 11.

<sup>6</sup> The contraction of these fibrin filaments will be described in a subsequent paper. A preliminary note was published in *Proc. Soc. Exp. Biol. and Med.*, 1930, 27, 618.

but also in rabbit and human blood. In rabbit blood the time consumed was 2 minutes and 80 minutes in human blood.

2. A second method is as follows: A red corpuscle is stretched by two opposite fibrin threads into a spindle form. During observation the spindle corpuscle becomes smaller, generally symmetrically, and the hemoglobin tint deepens until a spindle-shaped microcyte is formed (Fig. 1, Group E). The change may be complete in 3 minutes; it was observed in dog, rabbit and guinea pig.

At times one may observe repeatedly that the same spindle corpuscle becomes thinner and longer and then shorter and thicker. During the stretching period a few small projections may appear on a section of the corpuscular surface. After formation of the microcyte these projections appear as short, pointed crenations which disappear in a few minutes.

3. The third method was observed in a rabbit blood preparation 25 hours old. This blood had coagulated and a section of the peripheral clot as well as of the centre had sunk away from the cover-slip. Adjacent parts of the periphery exhibited round or oval red corpuscles arranged in a single layer like an epithelioid border. These corpuscles showed no dells, even on oblique illumination and measured  $6.5\mu$  in diameter. While observing this field, a stream of red corpuscles in single file began to flow with moderate speed from the periphery towards the serum centre of the drop. The corpuscles traversed a tortuous path, colliding with stationary corpuscle groups, leucocytes, fibrin networks or squeezing their way between these obstructions. During this obstacle race one or another of the red corpuscles was abruptly anchored in the fairway by a fibrin thread which suddenly appeared on one side of the corpuscle. The originally round or oval structure now elongated and retracted, swaying on its fibrin filament as it was buffeted in the narrow channel by the passage of companion corpuscles and serum. At times this fixation was but momentary; the fibrin thread ruptured and now a pear-shaped corpuscle hurried on its way, retracting during inspection first into a coarsely crenated form, then into a thorn-apple form with more numerous and more pointed crenations, and finally into a typical microcyte covered on all visible surfaces with still more numerous fine, thin spicules. These delicate spicules disappeared during observation and now a typical



microcyte  $5\mu$  in diameter, with smooth surfaces and dense hemoglobin tint had been formed (Fig. 2, Group G). The time consumed in the transition from pear shape to the coarsely crenated form may be less than 1 second; the next stages to the final microcyte may be completed in 5 to 10 seconds.

If the pear-shaped erythrocyte is fixed for a longer time in the stream bed, one observes that both the fibrin thread and the corpuscle elongate when passing cells brush by. During one of the recoveries from these distortions, a few crenations suddenly appear. These crenations become more numerous and finally when the fibrin anchor breaks, a coarsely crenated corpuscle rolls down the passageway. The whole process may be completed in 5 to 10 seconds (Fig. 2, Group H). This crenated form generally does not change to a microcyte during subsequent minutes.

The rapid changes described above were observed only when the corpuscle was subjected to distortion. If traumatism was lacking, if a corpuscle passed slowly through the channel and exhibited but little change in contour during its transit, this corpuscle preserved its round or oval shape. Moreover, the entire change of form exhibited by the corpuscles was produced during passage through the channel and then the structures maintained their attained shape in the quiet central serum pool for at least 30 minutes. In this central serum pool the forms were arranged roughly from the periphery to the centre as follows: Coarsely crenated or round corpuscles, finely crenated corpuscles and then microcytes.

The transformations described above were noted dozens of times during a 44 minute period of observation in one rabbit blood specimen. The same complete process was also seen in another rabbit preparation which was 2 hours old. Various stages of the process were also noted in human and guinea pig blood.

4. The fourth method of producing microcytes is this: The hemoglobin stroma of a corpuscle suddenly retracts or contracts with a jerk into a rounded, dense, yellow-brown mass which separates smoothly from a section of the surface pellicle and darts to one side of the red cell, leaving a space filled with clear liquid. At the same time the corpuscle may become pear-shaped as if anchored by an invisible fibrin thread (Fig. 2, Group I). The free section of the surface layer

now appears as a grayish white, fairly refractile and apparently doubly contoured pellicle which resembles the wall of a blood shadow. There is no observable change in that part of the surface layer which remains in contact with the hemoglobin mass. The whole process occurs with startling abruptness and is complete in a fraction of a second; it was directly observed only twice in my entire experience and in both instances occurred in preparations from two different rabbits. In one of them (Fig. 2, Group I) the corpuscle involved had been continuously watched for 77 minutes in order to observe the process of laking when the retraction took place. During the next 37 minutes the sheath of the microcyte became somewhat smaller, but remained pear-shaped and its contents clear (Fig. 2, Group I).

Although the process itself was only seen twice in action, its results were noted frequently in guinea pig, rabbit and human blood. In guinea pig blood this type of microcyte appeared within 30 minutes after making the hanging drop; in rabbit blood within 1 to 25 hours, and in human blood within 50 minutes.

In both guinea pig (Fig. 2, Group K, No. 4) and human blood, short fibrin threads were seen attached to the apex of the pear-shaped pellicle; in other instances however the pellicle showed no fibrin thread and no definite deformation of contour.

The liquid contents of the space between the microcyte and pellicle is not always clear, but may show a slight yellow tinge as if some hemoglobin had gone into solution.

Microcytes of the type described in this section are perhaps not formed solely from ordinary red corpuscles for apparently the same process may occur in fusing red corpuscles. In Fig. 2, Group K, Nos. 2 and 3, pairs of typical microcytes are sketched which are or were enclosed in a single pellicular sheath. The development of these microcyte pairs was not seen, but it seems legitimate to interpret them as examples of the Type 4 process occurring in fusing corpuscle pairs. It is interesting to note on the basis of this interpretation that the force of the stromatic contraction or recoil was so violent that rupture at the site of pellicular fusion occurred (Fig. 2, Group K, No. 2); furthermore, No. 3 demonstrates that the entire hemoglobin stroma may tear away from the surface layer, so that the resulting microcyte lies loose in a sheath like a coin in an old-fashioned purse.

*Reversibility of the Process Forming Microcytes.*—The reversion of a typical microcyte to a fairly normal erythrocyte was seen only once, but this single instance was especially valuable because the formation of this macrocyte by the process sketched in Fig. 1, Group E, had been directly observed. This single red cell therefore was first changed to a microcyte and then back to a fairly typical erythrocyte. The transformation occurred in a specimen of rabbit blood. After formation of the microcyte this structure suddenly moved in the field and now two fibrin threads at opposite sides became visible. During inspection, the microcyte elongated into a stubby spindle, due to the pull of the fibrin filaments. After a while the fibrin threads suddenly were seen to break at their peripheral attachment and roll up into two, fluffy, slightly refractile balls which came to rest on opposite sides of the cell. At the same time that the fibrin threads ruptured, the spindle microcyte swelled in its transverse surface diameters, became perfectly round and now exhibited a definite central delle. The hemoglobin tint was paler than that of the parent microcyte and the size of the corpuscle was slightly smaller than that of a normal erythrocyte (Fig. 2 Group F). It is therefore clear that the process of forming a microcyte of this type is reversible, and that the essential architecture of a red corpuscle is not destroyed by its compression into a microcyte.

*Stagnating Blood.*—No increased production of microcytes was seen in preparations of blood which had been previously allowed to stagnate for 5 to 10 minutes in its normal channels.

*Bone Marrow and Spleen.*—In hanging drop preparations made with Ringer solution or physiological saline, no increased formation of microcytes was seen. As in ordinary blood specimens, microcytes appeared within 3 to 6 minutes after making the suspension; most of them possessed numerous, very thin, short spicules and seemed to be formed *via* the crenation route. No attempt was made to follow the process in a single cell. The microcytes became more numerous with increasing age of the preparation.

#### DISCUSSION

In the following analysis of the chief morphological and functional manifestations observed in red corpuscles, it must be remembered that these changes were studied on blood which was coagulating or had

coagulated in a miniature, sealed, moist chamber at room temperature. No distinction is made between the various species of blood because the general processes appeared to be the same in all. It may be permissible to point out again that most of the experimental data were obtained from the guinea pig, rabbit and human subject and that the described procession of changes was directly observed in a selected cell or group of cells.

*Formation of Macrocytes and Microcytes.*—The evidence presented on preceding pages gives convincing proof for the first time to my knowledge, that erythrocytic macrocytes and microcytes form at room temperature in hanging drop preparations of normal, undiluted, coagulating blood. It is doubtful whether or not these changes occur under normal conditions in the capillary channels and sinuses of the body. The few experiments with stagnating blood and with bone marrow and spleen pulp reported previously are inadequate for a final answer, but as far as they go, they speak against this assumption. It may be noted however that the spleen and bone marrow experiments were not exactly comparable to the blood preparations, because physiological saline or Ringer solution had to be added; in addition all the spleens examined were in a state of strong contraction. It is conceivable that red corpuscles enfeebled by age, moving slowly through the crowded sinuses of the spleen might readily exhibit some of the changes described above (Fig. 2, Groups G and H). Similarly under pathological conditions, for example the various types of anemia, it is possible that some of the macrocytes and microcytes may be of peripheral origin; Nägeli's macrocytes<sup>7</sup> in chlorosis may be the result of a fusion between red corpuscles.

Since red corpuscles may fuse when apposed border to border, it is conceivable that the same fusion will take place when the flat surfaces of red corpuscles are touching each other. This process, however, I have never definitely seen. Numerous times red corpuscles of normal size and contour were encountered which had a hemoglobin tint as deep as that of microcytes. Possibly they were formed by the fusion of microcytes (Fig. 1, Group C) or by the process mentioned above.

*Membrane or Condensation Product.*—The observations made on the

<sup>7</sup> Nägeli, O., in Schittenhelm, A., *Handbuch der Krankheiten des Blutes und der blutbildenden Organe*, Berlin, Julius Springer, 1925, 1, 22.

formation and laking of macrocytes throw some additional light upon the old and much discussed problem whether a true, anatomical, delimiting membrane exists in the mammalian erythrocyte, or whether the surface layer is a condensation product of the stroma.<sup>8</sup>

Without entering into unnecessary detail concerning this perhaps academic problem, it seems that the observations recorded in this paper indicate that the surface layer is rather a condensation product of the stroma than a fixed, definite anatomical membrane. Thus when two corpuscles begin to fuse, the apposed, now intracorpuseular surfaces seem to separate within a few minutes into their constituent fibrils and become integral parts of the macrocyte stroma. The progress of this incorporation appears to be indicated by the gradual disappearance of the surface groove, of the septum itself, by the appearance of a round or slightly oval shape, and by the redistribution of hemoglobin so as to form a homogeneous tint when a dense microcyte fuses with a corpuscle of lesser hemoglobin concentration (Fig. 1, Group B). Upon laking, the macrocytic shadow reveals no septum if the fusion was complete; if incomplete, a partition separates the shadow into two compartments. Hemolysis at times discloses a septum when none was visible in the macrocyte.

In one interesting observation (Rabbit X 3-115) there was evidence that up to a certain point the process of changing the septum into stroma fibrils is reversible: an oval macrocyte  $9\mu$  long with a single septum was under observation until the septum became invisible and only a slight indentation of the outline still betrayed the pull of its fibrils. Upon hemolysis of this macrocyte, three round shadows appeared, each touching its two companions; the diameter of each shadow was  $4\mu$ . Here apparently the original surfaces of the three microcytes were reformed when hemolysis occurred. This observation, however, is unfortunately incomplete: the hemolysis was not observed directly; it took place while the eye was inspecting another section of the field. Upon return to the place formerly occupied by the macrocyte, the three microcyte shadows were found in its stead. No other noticeable change had occurred in this field.

An additional indication that the surface layer is a modified stroma, is the presence of a pigment, probably hemoglobin, in the surface layer itself: when two corpuscles fuse, the apposed surfaces present themselves as a whitish, moderately refractile septum which resembles the wall of a blood shadow. Incorporation of the septum apparently begins by removing the pigment bound to the surface

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<sup>8</sup> For a review of the literature see Michels, N. A., *Haematologica*, Recensiononi, 1931, 2, No. 3, 32 (reprint); Stewart, G. N., *J. Pharmacol. and Exp. Therap.*, 1909-10, 1, 74; Brodersen, J., in von Möllendorf, W., *Handbuch der mikroskopischen Anatomie des Menschen*, Berlin, Julius Springer, 1927, 2, pt. 1, 596; Krumbhaar, E. B., in Cowdry, E. V., *Special cytology*, New York, Paul B. Hoeber, 1928, 1, 279.

layer. The ready visibility of the extremely slender spicules often seen on crenated microcytes also indicates the presence of a pigment in the surface layer.

*State of Corpuscular Hemoglobin.*<sup>9</sup>—It is inferred by many investigators that hemoglobin exists in the corpuscle as a loose physico-chemical compound. In the present paper evidence has been presented that the hemoglobin of an intact corpuscle may be present in a bound, undissolved form (Fig. 2, Group I). In these observations the hemoglobin stroma was seen tearing away from the surface layer and contracting into a rounded dense brownish yellow mass, leaving a clear liquid between itself and the surface layer. The clear liquid possibly represents material which was present in the hemoglobin stroma interstices, mixed perhaps with serum components which filtered through the surface layer. Obviously no volume determinations of the various stages observed could be made.

*Hemolysis.*—The laking of macrocytes was observed numerous times and three different types could be distinguished. The fulminant type in which the hemoglobin is lost in a fraction of a second, has already been described. In the second type, the initial loss of hemoglobin is rapid; but then the process slows and many minutes pass before the macrocyte attains the same tint as the surrounding serum (Fig. 1, Group A). The third type is characterized by a slow, equable, loss of hemoglobin during 20 to 80 minutes.

The fulminant type is particularly interesting because of its startling abruptness and because the resulting shadow is at times paler than the background. Although the available data are insufficient to permit a full analysis yet a picture of the process may be constructed if these data are joined together by some assumptions. The observational

<sup>9</sup> For a general survey of hemolysis see Stewart, G. N., *J. Pharmacol. and Exp. Therap.*, 1909-10, 1, 49-121; Mond, R., *Protoplasma*, 1927, 2, 126-134; Ponder, E., *Brit. Med. J.*, 1927, 2, 295-298; Brinkman, R., in Bethe, A., von Bergmann, G., Emden, G., and Ellinger, A., *Handbuch der normalen und pathologischen Physiologie*, Berlin, Julius Springer, 1928, 6, 1st half, pt. 1, 567-585; Brodersen, J., in von Möllendorf, W., *Handbuch der mikroskopischen Anatomie des Menschen*, Berlin, Julius Springer, 1927, 2, pt. 1, 607; Krumbhaar, E. B., in Cowdry, E. V., *Special cytology*, New York, Paul B. Hoeber, 1928, 1, 392.

The limitations of the method employed in this work prevent the separation of hemoglobin and stroma in analyzing hemolysis.

facts in fulminant hemolysis are: (1) a slight, momentary, general jerk of the macrocyte precedes hemolysis; (2) this jerk does not deform or tear the surface layer; (3) after the jerk, the hemoglobin leaves the macrocyte in a fraction of a second; (4) the visible surface area of the macrocyte and the resultant shadow are the same; (5) the shadow is paler at times than the surrounding serum. Since it has been demonstrated that the hemoglobin stroma of a red corpuscle may retract or contract with a jerk (Fig. 2, Group I), it is justifiable to interpret the initial jerk as a sign of vigorous, general stromatic shortening. Since the macrocytic shadow contained little or no hemoglobin, it may be inferred that most of the hemolysis occurred at or in the surface layer. This supposition demands a concentration of the hemoglobin in a non-dissolved form at the internal surface of the macrocyte. Such a concentration could be readily produced if the hemoglobin stroma ruptured in the equatorial portion of the macrocyte, at the site perhaps where the original, component corpuscles fused, while its anchorage to the surface layer remained intact. Ruptures approaching this type are sketched in Nos. 2 and 3 of Fig. 2, Group K.<sup>10</sup> Under these conditions the retracting or contracting hemoglobin stroma would impinge with some force on the inner aspect of the surface layer and this force could drive the hemoglobin into the surrounding medium if solution of the hemoglobin occurred at or in the surface layer. Evidence of this force is seen in No. 2, Group K, where the surface layer was ruptured.

What the particular change in the surface layer is that brings about hemolysis cannot be answered with the data at hand.<sup>11</sup> It may be stated, however, that the surface layer reveals other changes which

<sup>10</sup> This type of tearing of the stroma was never seen directly, but its results are not infrequently noticed in hanging drop preparations. A slow, gradual concentration of hemoglobin into a peripheral ring was observed in ordinary red corpuscles and in microcytes, but never in macrocytes; in these instances apparently, a slow relaxation of the central stroma occurred without hemolysis. Ring-shaped concentrations of hemoglobin have been described and figured by Maragliano, E., and Castellino, E., *Z. klin. Med.*, 1892, 20, 427, Plate V, Series G, Fig. 7.

<sup>11</sup> See Ponder, E., *Brit. Med. J.*, 1927, 2, 295-298, for the various hemolytic systems; and Mond, R., *Protoplasma*, 1927, 2, 126, for the physicochemical basis in non-specific hemolysis.

indicate a loss of elasticity and flexibility. This increased rigidity develops rapidly and immediately precedes hemolysis. Thus the macrocyte may exhibit transitory, local, broad based bulgings of slight depth some minutes before fulminant hemolysis, indicating the presence of flexibility and elasticity, yet the initial general jerk caused no change in the contour, nor did the subsequent expulsion of hemoglobin produce any change in size of the visible surface area; in addition the surface layer lost its pigment and became at once grayish white and slightly refractile. All these observations point to alterations of the surface layer which may well be associated with one that induces hemolysis.

The other two types of spontaneous hemolysis observed in macrocytes are explicable on the basis of stroma contraction and intracorpuseular hemolysis plus diffusion, or only intracorpuseular hemolysis with diffusion may be operative. The initially rapid and then slow loss of hemoglobin may thus be caused by a contraction of the hemoglobin stroma towards the surface layer which is at first rapid enough to expel the dissolved hemoglobin forming at or in the surface layer, but which then slows sufficiently for the hemolytic process to progress from the surface layer to the intramacrocytic hemoglobin stroma so that dissolved hemoglobin is liberated in the macrocyte itself; this dissolved hemoglobin then diffuses out of the macrocyte. In this type of hemolysis no initial jerk of the macrocyte was seen, indicating that the stroma contraction if present was gradual in its onset. In the last type, it is probable that intramacrocytic hemolysis occurred from start to finish; this liberation of hemoglobin in a dissolved form appeared to be slow, for the macrocyte never looked transparent, but was always opaque.

*Volume Changes.*—No volume determinations of macrocytes were possible because these structures did not roll over so as to permit thickness measurements. Occasionally, it is true, a temporary, lenticular outline was observed, but I never could decide whether this represented a side view or a variation in the contractile state of the stroma. In macrocytic shadows also no depth measurements were feasible because of their stationary position. With microcytes, on the other hand, thickness could be measured a few times in rabbit blood. As reported on page 553 these microcytes on side view were



cylindrical, with rounded ends,  $5\mu$  long and 2.5 to  $3\mu$  thick and the diameter of the flat circular surface was generally  $5\mu$ . Calculated as a cylinder the volume would be  $53\mu^3$ ; the volume of the microcyte however would be less than this because of the rounded ends. The volume of a normal rabbit red corpuscle ( $7 \times 7 \times 2.25\mu$ ) calculated as a cylinder with flat ends would be  $86.6\mu^3$ ; allowing a 20 per cent deduction<sup>12</sup> for the rounded edges and the delle, the volume would be  $60.3\mu^3$ . It thus appears that the microcyte has definitely a smaller volume than a normal red corpuscle.

Since hemoglobin forms a considerable fraction of the corpuscular volume, the failure of a macrocyte to exhibit a decreased visible surface area after fulminant hemolysis requires explanation. Evidence has already been submitted which indicates an increased rigidity of the surface layer in the laking macrocyte and therefore the loss of hemoglobin could only be compensated by a collapse of the two flattened sides towards each other, or by the entrance of liquid serum components into the shadow or by a combination of these two processes. As the shadow was occasionally paler than the hemoglobin-stained background for a period of time after fulminant hemolysis had occurred, it seems probable that this pallor was caused by a collapse of the flattened sides so that a double thickness of the wall was seen. The gradual change of the shadow to the color of the background is perhaps attributable to entrance of the surrounding serum into the shadow through the permeable surface layer. It is regrettable that no side view of such shadows could be obtained.

*Elasticity, Flexibility, Selective Permeability.*—These qualities are generally attributed to the red corpuscle, particularly the surface layer, and with them as aids we may perhaps arrive at an explanation of some if not all of the changes sketched in Figs. 1 and 2, Groups D, E, F, G, H, I and K. It should be noticed that the capacity to elongate and shorten is not always the same in the surface layer and the hemoglobin stroma. In Groups D, E and I the entire corpuscle

<sup>12</sup> For volume determination of single red corpuscles see Welcker, H., *Z. rationell. Med.*, 1864, 19, series 3, 266; also Götze, R., *Z. Konstitutionslehre*, 1924, 9, 239. Welcker deducts 19.3 per cent from the cylinder volume as compensation for the rounded edges and the delle in human corpuscles; Götze, by a geometrical method, found 18.5 per cent in pig, and smaller values in horse, cattle, sheep and goat.

elongates and shortens as a whole while in Groups I and K practically only the hemoglobin stroma shortens vigorously and abruptly, the surface layer having lost most of this capacity. Furthermore the surface layer and hemoglobin stroma show local variations in extension and shortening which exhibit themselves as mamillary and papillary bulgings. In Groups G and H these papillae were apparently the result of repeated, transitory rises in intracorpuseular pressure. The papillae were short, broad based, with a blunt tip at first; more or less rapidly these coarse crenations may become progressively thinner, more pointed and, curiously enough, more numerous while the corpuscle decreases in size and assumes a deeper hemoglobin tint. Finally the resultant spicules disappear and now the surface layer has apparently shortened so as to present a perfectly smooth outline and surface.

The conditions under which a dense microcyte without dells enlarges to a fairly normal red corpuscle with a dells, are less easily accounted for (Fig. 2, Group F). The appearance of fibrin filaments indicates that the microcyte content was under tension and that the permeability for intracorpuseular fibrinogen<sup>13</sup> was locally increased. What rôle the stretching of the microcyte played in producing the final transformation cannot be stated. As no thickness measurements were possible, we do not know whether the increase in size was due to the entry of serum liquids or whether the surface layer and hemoglobin stroma relaxed, becoming wider and thinner without any significant volume change.

#### SUMMARY

In hanging drop preparations of normal blood from various species of animals including man, the following processes were directly observed for the first time:

1. The process of fusion between two red corpuscles so as to form a round or slightly oval macrocyte with normal hemoglobin content, a diameter of  $10\mu \pm$  and no dells.

These macrocytes appear to be slightly thicker in the centre than at the periphery. No thickness measurements could be made.

<sup>13</sup> Horino, K., *J. Biochem.*, 1928, 9, 437.

2. The process of fusion between two microcytes each  $5\mu\pm$  in diameter, so as to form a red corpuscle of approximately normal size but with dense hemoglobin and no dells.

3. The process of fusion between a red corpuscle and a microcyte, forming a macrocyte slightly larger than a normal red corpuscle and exhibiting a normal hemoglobin tint but no dells.

4. The process of microcyte formation from red corpuscles was seen occurring in four different ways: (a) By the intermittent compression of a single red corpuscle by the pull of three or more fibrin threads attached to the corpuscle. (b) By the steady, continuous compression of a single red corpuscle by the pull of two polar fibrin threads. (c) By intermittent rises of intracorpuseular pressure in a red corpuscle due to trauma. (d) By avulsion and retraction or contraction of the hemoglobin stroma from the intact surface layer of a red corpuscle.

Thickness measurements in single microcytes were made in rabbit blood.

5. The process of a typical microcyte with dense hemoglobin and no dells changing back to a fairly large red corpuscle with practically normal hemoglobin tint and with a dells.

6. Macrocytes were seen expelling their hemoglobin in a fraction of a second and turning into shadows which were often paler than the background, but without any decrease in the visible surface area or change in the contour.

7. Extensibility and retractibility (or perhaps relaxation and contraction) may be exhibited by the corpuscle as a whole, by local sections of both surface layer and stroma, or retractility (contractility (?)) may be shown by the hemoglobin stroma alone.

On the basis of observational evidence it is inferred that:

1. The surface layer of a red corpuscle is probably a condensed stroma rather than an anatomically defined membrane.

2. A pigment, probably hemoglobin, is present in the surface layer.

3. The surface layer of red corpuscles becomes rigid before or during spontaneous hemolysis.

4. Hemoglobin exists in an undissolved form in the intact red corpuscle.

5. The transverse diameter of microcytes ( $5\mu\pm$ ) represents the maximal shortening of which this diameter is capable in normal red

corpuscles of man, rabbit and guinea pig under the conditions studied.

6. Fusing red corpuscle are destroyed by repeated washings with Ringer solution.

7. In stagnating blood there is no increase in fusion forms, nor in the production of microcytes.

*Group A.*—Human blood (A.G.P.); X 4-87. Hemoglobin 80 to 90 per cent (estimated). 2.37. Hanging drop prepared; room temperature 25.8°C. 3.10. Fusing reds 13 $\mu$ ; constriction, septum well marked. Hb tint normal; no dells. 3.12. Septum gone; Hb as before; constriction marked; diameter no change; no dells. 3.13. Constriction shallower; no septum; Hb same. 3.15. Outline now smooth; no septum; Hb same; 13 x 9.1 $\mu$ . 3.18. Rounder; 11.7 x 9.7 $\mu$ ; Hb same; no septum; no dells. 3.23. Same. 3.25. Slightly smaller, 10.4 x 9.1 $\mu$ ; Hb same. 3.31. Same; 10.4 x 9.1 $\mu$ . 3.38. Same. 3.39. Perhaps slightly paler. 3.40. Quite pale; perhaps slight constriction on upper side. 3.44. Very pale; difficult to measure, 11.7 x 9.1 $\mu$ . 3.47. Same; room temperature 25.7°C. 3.49. 10.4 x 8.4 $\mu$ ; difficult to measure; slightly oval. 3.51. Same. 3.59. Barely perceptible; almost same color as background. 4.05. No longer seen.

*Group B.*—Black-gray rabbit, female, 2400 gm.; not pregnant; X 3-135. Hemoglobin (Dare) 66 per cent. Room temperature 22.6°C. 1.25. Hanging drop prepared. 1.38. Fusion of red corpuscle and microcyte; septum plain, with groove; Hb normal for each. 1.39. No septum visible; no dells. 1.43. Egg-shaped; no change in Hb. 1.44. Hb less than in original microcyte. 1.49. No change. 1.55. No change; oval. 1.57. Practically round; Hb same. 1.59. Again slightly oval; Hb perhaps slightly paler. 2.00. During observation pales abruptly; shadow same size. 2.01. Oval shadow.

*Group C.*—Same rabbit as in Group B. Same preparation. Same day. 2.10. Two microcytes, dense Hb, beginning to fuse; no dells; septum marked 10.4 x 5.8 $\mu$ . 2.12. Waist less marked, shifted towards one side; septum distinct; Hb dense 5.8 x 9.1 $\mu$ . 2.15. Septum barely visible; 6.5 x 8.5 $\mu$ . 2.17. Oval mass; dense Hb; no septum visible. 2.19. No change; Hb dense as in original microcyte. 2.22. Rounder; Hb same. 2.25. Slightly oval; Hb same. 2.27. Practically round; no dells, 7.8 x 8.4 $\mu$ ; Hb same; no septum. 2.29. No change. 2.31. No change. 2.32. Suddenly pales. 2.32½. Shadow 7.8 x 8.4 $\mu$ ; round; same color as background; room temperature 21.8°C.

*Group D.*—Guinea pig, male, 610 gm.; X 3-24. Hemoglobin (Dare) 106 per cent. 1.25. Hanging drop prepared. 1.26. Spindle and pear-shaped reds in periphery of drop. 1.29. See description of a pear-shaped red corpuscle changing to a microcyte on page 556. The fibrin threads at the angles of the corpuscle were not equally visible. The various shapes were maintained for a short period of time and the change then occurred with a snap. No measurements made; no time. 1.30. Typical microcyte.

*Group E.*—Guinea pig, male, 650 gm.; X 3-28. Hemoglobin (Dare) 109 per cent. 9.40. Hanging drop prepared; ear vein. 9.41. Angulated reds in periphery of drop; no fibrin threads visible at angulations. 9.45. Spindle red observed becoming smaller; decrease symmetrical; Hb tint becoming deeper; no movement. 9.47. Spindle red smaller; fibrin thread at upper pole longer, point of attachment not visible; fibrin thread at lower pole visible for short distance. 9.49. Now a stubby spindle microcyte; dense Hb; no dells; total length from tip to tip 10.4 $\mu$ , width 5.2 $\mu$ .

The same process occurred in a few seconds in Rabbit X 3-40; this blood preparation was 65 minutes old.

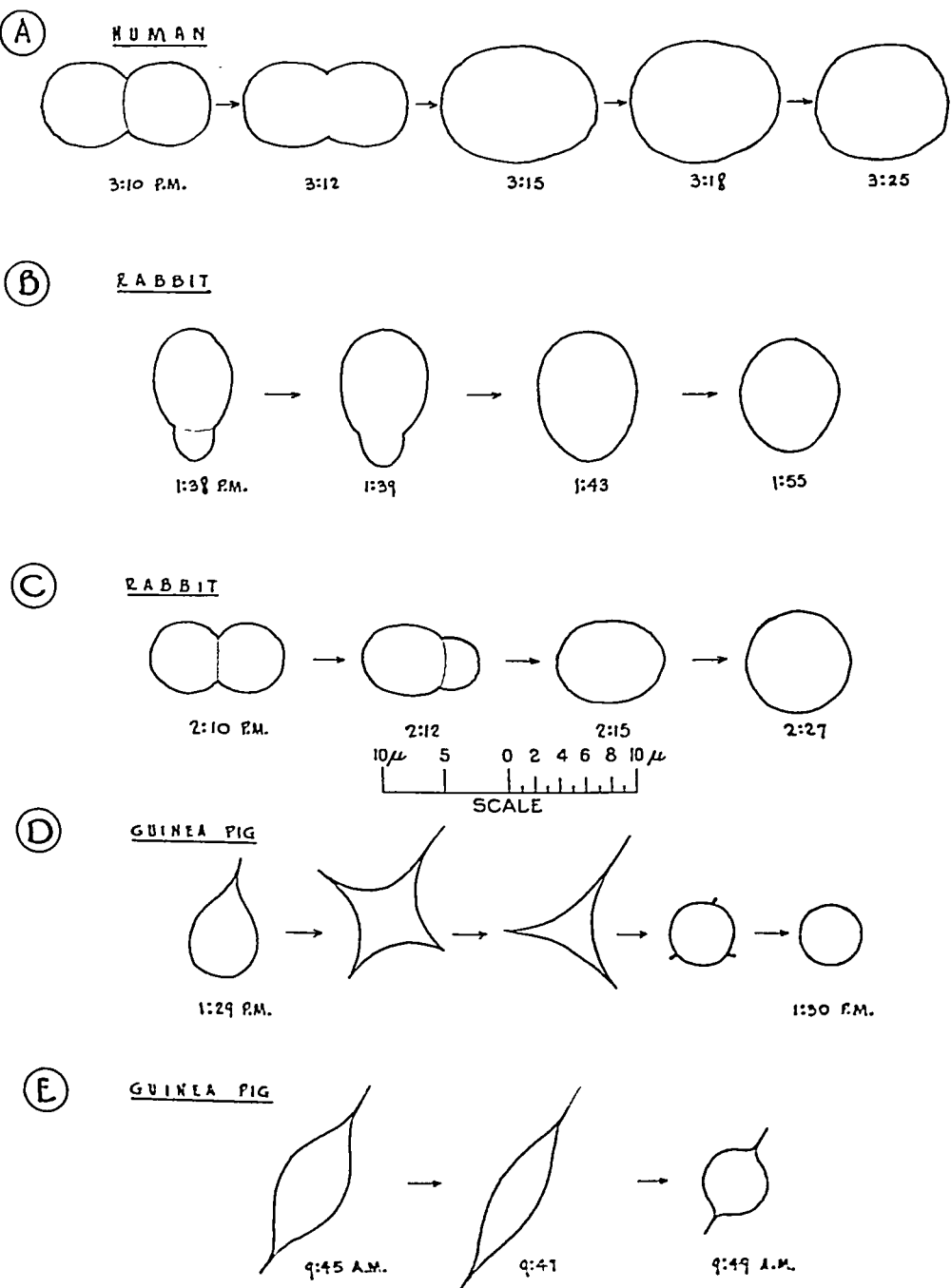


FIG. 1

FIG. 2

*Group F.*—Red rabbit, male, 2800 gm.; X 3-15. Hemoglobin (Dare) 90 per cent. Room temperature 65°F. 9.32. Hanging drop; ear vein. 9.34. Reds lake in periphery of drop. 9.37. Spindle reds; fusing reds; occasionally three reds fuse. 10.20. Formation of a typical microcyte from a spindle red observed and sketched (omitted here; same as in Group E). This microcyte was observed changing back to fairly large red corpuscle with a delle; see description on page 560. No time for measurements.

*Group G.*—Gray rabbit, female, 2600 gm.; not pregnant apparently; X 3-140. Hemoglobin (Dare) 72 per cent; blood specimen 25 hours old. 3.00 to 5.00 p.m. Reds round or pressed together in periphery; no delle;  $6.5\mu$  in diameter. Sudden flow of red corpuscles in single file towards middle of drop. Red corpuscles seen changing to crenated forms and then to typical microcytes in 10 seconds or less. See description on page 557.

*Group H.*—Same preparation. Pear-shaped reds whose fibrin attachment did not rupture immediately after fixation in the stream bed. See description on page 558.

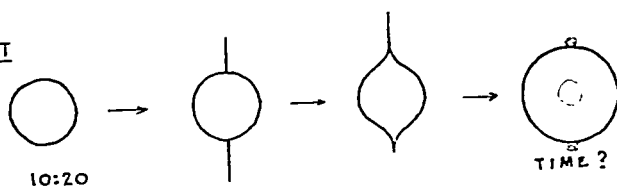
In Groups G and H no measurements were made because the changes were too rapid. All the transformations sketched in G and H were seen dozens of times in this preparation in as many different cells.

*Group I.*—Gray rabbit, male, 2800 gm.; X 4-7; Hemoglobin (Dare) 85 per cent. Room temperature 24.0°C. Preparation 25 hours old. 3.29. Red  $7.8\mu$  diameter; no definite delle; good Hb. 3.30-3.47. No definite change. 3.47. Outline shows slight slow bulgings; no quivering;  $7.8\mu$ . 3.47-3.59. Same; Hb as before; no delle;  $7.8\mu$ . 4.03-4.30.  $7.8\mu$ . 4.34. Oval,  $7.8 \times 6.0\mu$ . 4.42. During observation jerk occurred and Hb shot as a yellow ball to one side of red; clear space between Hb ball and wall; pear shape; process complete in fraction of second;  $7.8\mu \times 4\mu$ ; see page 558. 4.46. Shell closer to microcyte. 4.47. No fibrin thread visible; Hb ball dense;  $5.2 \times 4\mu$ . 4.55. Still as before; clear space, not tinted; no spicules. 5.03. No change. 5.13. In same field two other reds with Hb ball torn away from pellicle; not present before. 5.19. No change; room temperature 25.9°C.

*Group K.*—Guinea pig, male, 950 gm.; X 1-12. Hemoglobin (Dare) 106 per cent. Four different corpuscles. 9.38. Hanging drop; ear vein. 10.05. No. 1, red with dense Hb, oval,  $8\mu$  long; Hb partly separated from wall; Hb mass shows median constriction. Lost. 10.10. No. 2, two round, dense microcytes each  $4.8\mu$ , connected by a thin membrane shred; upper membrane cuffed, angulated, as if torn. 10.31. No. 3, two oval, dense microcytes in hour-glass membrane; space between microcytes and wall slightly yellowish;  $4.8\mu$ . No. 4, oval dense microcyte surrounded by a pellicle, with short fibrin thread; space slightly yellow.

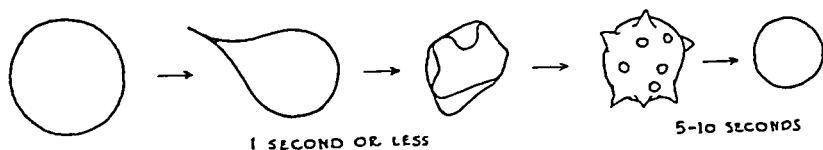
(F)

RABBIT



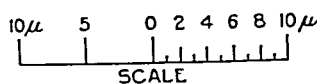
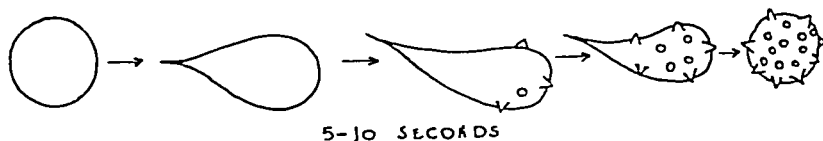
(G)

RABBIT



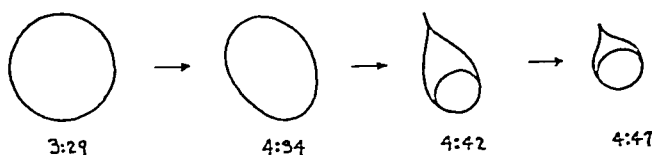
(H)

RABBIT



(I)

RABBIT



(K)

GUINEA PIG



FIG. 2





## STUDIES ON IMMUNITY TO SWINE INFLUENZA

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## INTRODUCTION

It has previously been shown (1) that the bacterium, *H. influenzae suis*, and a filtrable virus are essential to the production of influenza in swine. When administered separately intranasally, *H. influenzae suis* is completely non-pathogenic, while the filtrable virus when similarly introduced induces a very mild and scarcely recognizable illness that has been designated as the "filtrate disease" in distinction from swine influenza which results when virus and organism are administered intranasally together. The present paper describes studies dealing with the immunizing properties of each of the etiological components.

*Infectious Materials Used*

As in our earlier experiments (1, 2), *H. influenzae suis* was grown upon plain agar slants to the condensation water of which had been added approximately 0.75 cc. of sterile defibrinated horse blood. On such media the growth of the organisms was limited largely to the bloody fluid at the base of the slant and a narrow zone of agar immediately above. A stock culture isolated in December, 1928, and designated as 451, has been used in all experiments recorded in this paper. Only the bloody condensation fluid of cultures was used in inoculating the swine in the experiments to be reported.

The swine influenza virus used was either a Berkefeld N or a Berkefeld V filtrate of lung, bronchial lymph nodes, and bronchial exudate from a fresh case of the disease. Animals to be used as a source of virus were killed on the 3rd or 4th day of their illness. Pathological material to be filtered was minced with scissors and ground fine in a mortar with sterile sand. A suspension of approximately 5 per cent was made in infusion broth, pH 7.3, shaken by hand for 10 minutes, and then centrifuged. The supernatant fluid was removed and filtered through a Berkefeld candle, and the resulting filtrate served as a source of virus.

In testing swine for immunity induced by previous inoculations of virus or

TABLE I  
*The Effect of H. influenzae suis and Swine Influenza Virus Administered Separately upon the Subsequent Susceptibility of Swine to Influenza*

Swine No.	Weight at beginning of experiment	Preliminary treatment		Inoculation to test immunity			Remarks
		Inoculated intranasally with	Result	Length of time after preliminary inoculation or exposure	Inoculated intranasally with	Result	
	lbs.			days			
922	19	2 cc. 24 hr. culture HIS* + 8 cc. infusion broth	No illness	18	5 cc. 10% suspension dried and glycerolated influenza virus + 0.2 cc. 48 hr. culture HIS	Swine influenza	No immunity
919	15	8 cc. Berkefeld N filtrate of influenza virus	Filtrate disease	18	"	No illness	Immune
920	13	"	"	18	"	"	"
921	17	"	"	18	"	"	"
923	16	2 cc. 24 hr. culture HIS + 8 cc. Berkefeld N filtrate of influenza virus	Swine influenza				To control effectiveness of organism and virus in inducing swine influenza
959	21	Control	Control		5 cc. 10% suspension dried and glycerolated influenza virus + 0.2 cc. 48 hr. culture HIS	Swine influenza	
1067	45	0.5 cc. 72 hr. culture HIS + 9 cc. defibrinated swine blood	No illness	11	9 cc. light suspension dried and glycerolated influenza virus + 1 cc. 40 hr. culture HIS	"	No immunity
1125	26	Control	Control		"	"	"

1155	50	1 cc. 48 hr. culture H1S + 9 cc. physiological saline	No illness	12	14 cc. light suspension dried and glycerolated influenza virus + 1 cc. 24 hr. culture H1S	Swine influenza	No immunity
1156	56	Control 2 cc. Berkefeld N filtrate of influenza virus Infected by pen exposure to filtrate disease	Control	31	10 cc. suspension lung, lymph nodes, and bronchial exudate from influenza-sick swine	No illness	Immune
1077	21						
1082	23	Control Infected by pen exposure to filtrate disease	Control	24	8 cc. suspension lung, lymph nodes, and bronchial exudate from influenza-sick swine	Swine influenza	Immune
1060	45						
897	17	Control Infected by pen exposure to filtrate disease	Control	42	4.5 cc. suspension glycerolated influenza virus + 0.5 cc. 48 hr. culture H1S	No illness	Immune
951	16						
1130	29	Control Infected by pen exposure to filtrate disease	Control	10	5 cc. light suspension dried and glycerolated influenza virus + 0.5 cc. 48 hr. culture H1S + 10 cc. physiological saline	Swine influenza	Immune
1136	35						
1070	59	Control 5.5 cc. Berkefeld V filtrate of influenza virus	Control	11		No illness	Immune
1135	40						

• H1S = *H. influenzae suis*.

organism, where fresh infectious material was not available at the time, glycerolated and dried materials were used. It has previously been shown (1) that the swine influenza virus can be stored for at least 41 days in 50 per cent glycerol or 54 days when frozen and dried by Swift's method (3). *H. influenzae suis*, however, does not survive glycerolation and its survival in dried material is irregular. Therefore in the experiments recorded in this paper in which glycerolated or dried virus was used, small amounts of cultures of *H. influenzae suis* have been added to the suspensions of virus just before using.

*The Effect of H. influenzae suis and Swine Influenza Virus, Administered Separately, upon the Susceptibility of Swine to Influenza*

As shown in Table I, three swine (922, 1067, 1155) which were inoculated intranasally with *H. influenzae suis* showed no evidence of illness following the inoculation and also were fully susceptible to swine influenza when tested later by intranasal inoculation with suspensions containing swine influenza virus and *H. influenzae suis*.

Five swine (919, 920, 921, 1070, 1077) were inoculated intranasally with Berkefeld filtrates containing swine influenza virus and all developed the mild filtrate disease. When inoculated later with the mixture of virus and organism they were found to be completely immune to swine influenza.

There was a possibility that dissolved products of *H. influenzae suis*, which could conceivably be present in infectious Berkefeld filtrates, might account, at least partially, for this development of immunity in filtrate-infected swine. To test this possibility three other swine (897, 1082, 1130) were infected with the filtrate disease by placing them in the same pens with animals inoculated with infectious Berkefeld filtrates. By this method of infection they received virus free from any trace of *H. influenzae suis* protein, and like the swine infected by intranasal inoculation, they were found subsequently to be immune to swine influenza.

The experiments recorded in Table I indicate that an attack of the filtrate disease, whether induced by intranasal inoculation with filtered virus or by exposure, confers an active immunity to swine influenza as induced by the concerted action of *H. influenzae suis* and the filtrable virus of swine influenza. The administration intranasally of *H. influenzae suis* alone induces no demonstrable immunity to swine influenza. These data indicate that the filtrable virus is of primary etiological significance and that *H. influenzae suis* plays only a secondary and contributory rôle in the clinical entity known as swine influenza.

*Neutralization of the Swine Influenza Etiological Complex by Serum of Animals Convalescent from the Filtrate Disease*

It has been previously shown (1) that the blood serum of a hog convalescent from swine influenza when mixed with an infectious suspension and administered intranasally to a susceptible animal was capable of preventing infection. It seemed of interest, therefore, to determine whether both of the etiological components were essential to the

TABLE II

*Neutralization of the Swine Influenza Etiological Complex by Serum of Animals Convalescent from the Filtrate Disease*

Swine No.	Weight	Inoculated with			Result	Remarks
		Infectious suspension	Filtrate disease convalescent serum			
			Source	Amount		
1128	36 lbs.	5 cc. light suspension dried and glycerolated influenza virus + 0.5 cc. 48 hr. culture HIS	Swine 1077 and 1082 pooled	10 cc.	No illness	Found subsequently to be immune
1132	29	" "	" "	10	" "	Autopsy negative
1135 (control)	40	" "	10 cc. physiological saline		Swine influenza	Autopsy typical of swine influenza

generation of this neutralizing property of convalescent serum, or whether, as in the active immunization just discussed, the filtrable virus alone was sufficient.

Filtrate disease convalescent serum was obtained by bleeding two hogs, from the tail, 24 and 31 days after their infection with the swine influenza virus. These sera were freed from bacteria by Seitz filtration and then combined in equal quantities. The procedure was as follows: To 5 cc. of a light suspension of dried and glycerolated swine influenza virus was added 0.5 cc. of the bloody condensation

fluid of a 48 hour culture of *H. influenzae suis*. 10 cc. of the filtrate disease convalescent serum was added to this mixture, and after standing at room temperature for 1 hour, it was injected intranasally into the test swine, 15.5 cc. into each animal. The control animal received the same amount of infectious suspension to which 10 cc. of sterile physiological saline had been added. The results are recorded in Table II.

The potency of the infectious suspension was evidenced by the clinical and autopsy picture presented by the control. The neutralization was apparently complete for Swine 1132 which developed no clinical evidence, and, when autopsied on the 7th day after inoculation, showed no pathological evidence of swine influenza. Swine 1128 also at no time appeared ill but on the 3rd day after inoculation it had a temperature of 40°C. This is the lower limit of what we have considered a fever temperature in swine. 2 weeks after its initial inoculation it was found to be immune to swine influenza. It thus seems likely that in this animal the neutralization was not quite complete, otherwise the animal would not only have shown no illness but have remained fully susceptible to infection with swine influenza. A smaller dose of infectious suspension or a larger dose of convalescent serum would probably have resulted in a mixture as neutral for Swine 1128 as the one employed was for Swine 1132.

As in the experiments dealing with the immunity to swine influenza conferred by an attack of the filtrate disease, the neutralization of infectious suspensions, capable of inducing characteristic swine influenza, by serum from swine convalescent from the filtrate disease again indicates the primary etiological significance of the filtrable virus and the secondary contributory rôle of *H. influenzae suis*.

#### *Failure of the Swine Influenza Virus to Induce Illness when Administered Intramuscularly*

In these experiments only glycerolated swine influenza virus was used. The cultures of *H. influenzae suis* were of the type used in the experiments previously described in this paper. Glycerolated virus was prepared as follows:

Portions of atelectatic or pneumonic lung of approximately hickory nut size and pieces of the edematous bronchial lymph nodes of somewhat smaller size were taken from swine infected with influenza that had been killed on the 3rd day of their illness. These pieces of tissue were placed in 50 per cent glycerol-physiological salt solution and stored at refrigerator temperature for at least 6 days before use in these experiments. To prepare infectious suspensions from tissues stored in this

way, pieces were cut with sterile scissors from the stored material. These were washed in three changes of sterile physiological saline and then ground in a mortar with sand and physiological saline to make approximately a 5 per cent suspension. Such suspensions were allowed to stand undisturbed for a few minutes and the supernatant fluid, when decanted, served as the infectious suspension.

Six swine were inoculated intramuscularly with glycerolated virus. Four of these animals were converted artificially into carriers of *H. influenzae suis* by intranasal inoculation with cultures of this organism. Experiments, which will be reported in detail later, demonstrated that swine receiving *H. influenzae suis* in this way carry the organisms in their respiratory tracts for at least 3 days. The organisms thus carried, while innocuous in themselves, maintain their full potential pathogenicity because when virus alone is administered to such animals swine influenza instead of the filtrate disease results. A carrier state was established in the four above mentioned swine because, should the virus have proven pathogenic when administered intramuscularly, swine influenza as induced by organism and virus together would have been easier to recognize than the filtrate disease induced by the virus alone. In this way the presence of *H. influenzae suis* in the respiratory tract served as an indicator for the invasion of the respiratory tract by swine influenza virus. The data for the six swine inoculated intramuscularly with glycerolated virus together with those for the control swine receiving the virus intranasally are recorded in the first four columns in Table III. It is there shown that while the three controls which received glycerolated influenza virus and *H. influenzae suis* intranasally all developed swine influenza, none of the six swine inoculated with the virus intramuscularly developed any recognizable illness in spite of the fact that four of them were carrying *H. influenzae suis* in their respiratory tracts. One of these animals, Swine 1146, killed 4 days after inoculation, was completely negative at autopsy for any pathology of swine influenza. Of the remaining five, saved to test for immunity, three were given two subsequent intramuscular inoculations of glycerolated influenza virus and showed no reaction or evidence of illness following either of these.

The above data, summarized in the left portion of Table III, indicate that swine influenza virus given intramuscularly is incapable of inducing filtrate disease, or swine influenza, in animals converted artificially into carriers of *H. influenzae suis*. The suggestion derivable from these experiments is that the swine influenza virus is effective in inducing disease only when introduced directly into the respiratory tract, and in this respect is similar to certain other viruses, some of which regularly infect only when introduced into the epidermis (dermatotropic viruses) and others only when introduced directly into nervous tissue (neurotropic viruses).



TABLE III  
*The Effect of Intramuscular Injection of Swine Influenza Virus*

Swine No.	Weight lbs.	Inoculation and route	Result	Inoculation to test immunity			Remarks
				Length of time after last inoculation	Inoculated intranasally with	Result	
1146	51	10 cc. glycerolated influenza virus i.m.* and 0.5 cc. 40 hr. culture HIS in 10 cc. physiological saline i.n.	No illness	days			Autopsy negative
1147	61	10 cc. glycerolated influenza virus i.m. and 2 similar subsequent inoculations controlled by Swine 1154	" "	14	14 cc. light suspension dried and glycerolated influenza virus + 1 cc. 24 hr. culture HIS	No illness	Immune
1148	63	10 cc. glycerolated influenza virus + 0.5 cc. 40 hr. culture HIS i.n.	Swine influenza				Control for infectivity of virus used on Swine 1146 and 1147 when administered i.n.
1149	77	0.5 cc. 48 hr. culture HIS + 4.5 cc. physiological saline i.n., 10 cc. suspension glycerolated influenza virus i.m., and 2 similar subsequent inoculations with glycerolated influenza virus i.m.	No illness	14	14 cc. light suspension dried and glycerolated influenza virus + 1 cc. 24 hr. culture HIS	No illness	Immune
1150	73	" "	" "	14	" "	" "	"

1151	40	10 cc. suspension glycerolated influenza virus + 0.75 cc. 48 hr. culture H1S i.n.	Swine influenza			Control for infectivity of virus used on Swine 1149 and 1150 when administered i.n.
1156	56				14 cc. light suspension dried and glycerolated influenza virus + 1 cc. 24 hr. culture H1S	Control for immunity shown by Swine 1147, 1149, and 1150
1088	79	0.5 cc. 44 hr. culture H1S + 10 cc. physiological saline i.m., and 10 cc. suspension glycerolated influenza virus i.m.	No illness	15	10 cc. suspension dried and glycerolated influenza virus + 0.5 cc. 44 hr. culture H1S	Immune
1100	57	10 cc. suspension glycerolated influenza virus i.m.	" "	15	" "	"
1172	33	0.5 cc. 44 hr. culture H1S + 10 cc. suspension glycerolated influenza virus i.n.	Swine influenza			Control for infectivity of virus used on Swine 1088 and 1100 when administered i.n.
1175	19				10 cc. suspension dried and glycerolated influenza virus + 0.5 cc. 44 hr. culture H1S	Control for immunity shown by Swine 1088 and 1100

\* i.m. = intramuscularly; i.n. = intranasally. H1S = *H. influenzae suis*.

*Immunity Following the Intramuscular Administration of Swine Influenza Virus*

The five animals mentioned above, which were saved to test for immunity, were inoculated intranasally with *H. influenzae suis* mixed with dried and glycerolated swine influenza virus. Swine 1147, 1149, and 1150 had received three intramuscular injections of glycerolated virus prior to their test for immunity while Swine 1088 and 1100 had received only one. All five were found to be completely immune to infection with material which in control swine induced clinically and pathologically characteristic swine influenza.

These experiments are summarized in the right portion of Table III and indicate that swine influenza virus given intramuscularly immunizes hogs against swine influenza without inducing any evidence of illness. So far as can be judged, a single intramuscular injection of glycerolated swine influenza virus confers just as satisfactory an immunity as do three injections.

It is not believed that intranasal inoculation with *H. influenzae suis* contributed to the immunization, in view of the fact that immunity developed whether it was administered or not. Swine 1147 and 1100 which received no *H. influenzae suis* developed as satisfactory an immunity as did the three animals which received the organism intranasally.

DISCUSSION

Evidence derived from the experiments reported in this paper indicates that, of the two components essential to the production of influenza in swine, the filtrable virus is of primary importance while *H. influenzae suis* plays only a secondary or contributory rôle. *H. influenzae suis* administered alone intranasally to swine induced neither illness, as had previously been established (2), nor immunity to swine influenza. The filtrable virus of swine influenza, on the other hand, while capable alone of inducing only the mild filtrate disease, established a solid immunity to swine influenza as induced by the mixture of virus and organism. These findings are supported by the observation that convalescent serum from swine that had suffered only the filtrate disease was capable of neutralizing the combined etiological complex of organism and virus. These results accord with the expected action of two agents of unequal etiological importance but both essential for the production of a disease.

The swine influenza virus showed a certain tissue specificity in that it was found to be incapable of inducing illness when administered intramuscularly to swine although it was uniformly infective when introduced into the respiratory tract. This fact suggests that the swine influenza virus bears a relationship to tissues of the respiratory tract like that of dermatotropic viruses to the skin or of neurotropic viruses to the central nervous system.

It was furthermore of interest, and perhaps of practical value, to note that swine inoculated intramuscularly with the swine influenza virus, while failing to become ill, nevertheless developed an immunity to swine influenza. Under the conditions of laboratory experimentation, intramuscular inoculation of swine with glycerolated swine influenza virus constituted a safe and satisfactory method of immunization. It is possible that this observation can be applied in developing a method of immunization against the disease for use in the field.

#### SUMMARY

Of the two etiological components of swine influenza, only the filtrable virus possessed immunizing properties. *H. influenzae suis*, while essential to the production of the disease, played only a secondary and contributory rôle and, alone, conferred no immunity. Serum of swine convalescent from the filtrate disease neutralized the swine influenza etiological complex of organism and virus. Intramuscularly administered swine influenza virus was incapable of inducing illness but did render hogs immune to swine influenza. It is suggested that a specific relationship, as regards infectivity, exists between the swine influenza virus and the tissues of the respiratory tract.

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## STUDIES ON THE SHWARTZMAN PHENOMENON

### I. DETOXIFICATION OF MENINGOCOCCUS CULTURE FILTRATES

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The conversion of toxin into toxoid has been studied by Ehrlich (1) and Ramon (2-4) for diphtheria toxin, by Behring and Ransom (5), Lowenstein (6), and von Eisler and Lowenstein (7) for tetanus toxin, by Weinberg and Goy (8) for the toxins of the *B. botulinus* and of the gas gangrene anaerobes (*i.e.*, *B. oedematiens*, etc.). Ramon (9) has also converted abrin and cobra venom into toxoids. The change was observed to occur spontaneously (Ehrlich (1), Behring and Ransom (5)), and also could be induced artificially by means of various physical and chemical agents; namely, for tetanus toxin iodine trichloride (Behring and Ransom (5)), formalin (0.1 to 0.2 per cent) and exposure to a Nernst lamp (Lowenstein (6)), formalin (0.1 to 0.2 per cent) and slight heat (30°C.) (von Eisler and Lowenstein (7)); and for diphtheria toxin formalin (0.3 to 0.4 per cent) and heat (40-42°C.) (Ramon (4)). By similar procedures (formalin and heat) abrin, cobra venom (Ramon (9)), and toxins of the *B. botulinus* and the gas gangrene anaerobes (Weinberg and Goy (8)) have also been converted into toxoid states.

These studies elaborated the concept of a toxoid first announced by Ehrlich. In its completed form, the definition of a toxoid is that it is a toxin so altered that the toxicity is decreased whereas the antibody-combining capacity and the antigenicity are essentially undiminished. It has been observed (Lowenstein (6), Ramon (4)) that the antigenicity of tetanus and diphtheria toxoids varies with the potency of the mother filtrates. It can be estimated by the antitoxin-combining capacity, to which it is directly proportional.

By means of the phenomenon of local skin reactivity to bacterial filtrates, Shwartzman (10-13) has demonstrated the existence of a new

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category of bacterial exotoxins. They are filterable and best obtained under conditions of insignificant cell autolysis. The potency of a filtrate does not depend solely on the number of bacterial cells in the culture from which it is obtained. The toxins are highly antigenic. The potency of the factors necessary for the phenomenon can be accurately titrated. They are neutralizable in multiple proportions by specific antisera. Within the same bacterial species, there may be considerable variation in the potency of filtrates obtained from cultures of various strains. In a given filtrate, the potency seems to parallel the lethal effect on rabbits.

The demonstration of exotoxins of a new type in a large number of unrelated organisms as the members of the *coli*-typhoid-dysentery group, the meningococcus, the Pneumococcus Type III, the *Streptococcus haemolyticus-scarlatinae* (Shwartzman (12)), the gonococcus (Shwartzman (14)), the *Bacillus influenzae* (*Haemophilus influenzae*) (Frisch (15)), the *Bacillus pertussis* (Gross (16)), and the tubercle bacillus (Bieling (17)) was of great importance.

It was deemed of interest to determine whether this type of bacterial toxin could be converted into a toxoid state. With this aim in view, the following experiments were undertaken.

### *Methods and Materials*

These experiments were performed on rabbits which had been employed once within the previous week for the elicitation of the Shwartzman phenomenon, receiving for this purpose a variety of materials. Only those rabbits were selected in which the phenomenon had failed to occur. Unpublished experiments suggest that such rabbits may be more sensitive to the Shwartzman phenomenon than stock rabbits. Further experiments upon this point are now under way.

*Toxins.*—(a) Meningococcus “agar washings” toxic filtrates were prepared and titrated as described by Shwartzman (18, 19). Filtrate 1409 was prepared from meningococcus Strain 123, Group I; Filtrate 1675 from meningococcus Strain 383, Group II; Filtrate A52 was pooled from Filtrates 1233A, 1233B, 1233C, and 1233D, prepared from meningococcus Strains 44B Serum 8, 44B Serum 13, 44B Serum 15, and 44B Serum 16, respectively, all Group III (Shwartzman (20)). Meningococcus Filtrate 1675 was kept in the refrigerator at 6°C. from June 16, 1931, to Dec. 18, 1931 (time of retitration). The titers of the preparations are indicated in Table I.

(b) 1. 147 cc. of meningococcus “agar washings” toxic Filtrate 1409 plus 3 cc. of 20 per cent formalin to make a total volume of 150 cc. with a final concentration of 0.4 per cent formalin were placed in a ground glass-stoppered bottle, sealed

with paraffin, and kept in an incubator at 37°C. for 35 days. The resulting preparation is referred to as meningococcus Toxoid 1409A.

2. 2 cc. of 40 per cent formalin were added to 95 cc. of meningococcus Toxoid 1409A, thus making a final total concentration of 1.2 per cent formalin. This mixture was placed in a ground glass-stoppered bottle, sealed with paraffin, and kept in an incubator at 37°C. for 3 weeks (meningococcus Toxoid 1409B).

3. 110 cc. of meningococcus "agar washings" toxic Filtrate A52 plus 2.75 cc. of 40 per cent formalin (to make a final concentration of 1 per cent formalin) were placed in a ground glass-stoppered bottle, sealed with paraffin, and kept in an incubator at 37°C. for 23 days (meningococcus Toxoid A52).

*Sera*.—1. Antimeningococcus Serum H251 was obtained from Horse 8.

Immunization was started Oct. 13, 1930. The details of a similar course of immunization have been described elsewhere (Shwartzman (21)). In general, this consisted of the following: For 6 weeks, the horse received only weekly, subcutaneous injections of *B. coli* toxin. Thereafter, an intravenous injection of heat-killed *B. coli* vaccine was made with each subcutaneous injection of *B. coli* toxin, the dosage of both gradually increasing. After 6 weeks, polyvalent meningococcus toxin was added in the material for subcutaneous injection and meningococcus vaccine in that for intravenous injection. Finally the animal received intravenously incubated mixtures of meningococcus toxin and anticoli horse serum. Bleeding H251 was performed Mar. 10, 1931.

2. Serum H230 was obtained from Horse 60 which was immunized in order to obtain the auxiliary antibody (Shwartzman (21)).

Immunization was begun June 27, 1930. The animal received weekly subcutaneous injections of polyvalent *B. coli* toxin, of gradually increasing dosage. Intravenous injections were started Sept. 12, 1930. They consisted of increasing amounts of polyvalent heat-killed *B. coli* vaccine. In the last 5 weeks of immunization, 3 intravenous injections were made of incubated mixtures of polyvalent *B. coli* toxin and anticoli horse serum. The course of immunization lasted 8 months with one period of rest for 3 weeks. Bleeding H230 was performed Feb. 11, 1931.

3. Serum H290 was a later bleeding from Horse 60. After 2 weeks' rest, immunization was continued. The animal received 4 subcutaneous injections of polyvalent *B. coli* toxin, 2 intravenous injections of *B. coli* vaccine, and 2 intravenous injections of incubated mixtures of *B. coli* toxin and Serum H230. Bleeding H290 was performed Apr. 28, 1931.

*Neutralizations*.—Neutralizations of the toxins and toxoids by the horse sera were carried out as described by Shwartzman (19).

*Agglutinations*.—Agglutinations were carried out with standard suspensions of live 24 hour cultures of meningococci. Mixtures of 0.25 cc. of bacteria and 0.25 cc. of serum were kept in a water bath at 37°C. for 18 hours and then read promptly.

*Precipitations*.—Mixtures were made of 0.2 cc. serum and 0.2 cc. toxoid, 0.2 cc. serum and 0.05 cc. toxoid, 0.05 cc. serum and 0.05 cc. toxoid, and 0.05 cc. serum and 0.2 cc. toxoid. The mixtures were similarly incubated. Precipitations were also carried out using toxin as precipitinogen.



category of bacterial exotoxins. They are filterable and best obtained under conditions of insignificant cell autolysis. The potency of a filtrate does not depend solely on the number of bacterial cells in the culture from which it is obtained. The toxins are highly antigenic. The potency of the factors necessary for the phenomenon can be accurately titrated. They are neutralizable in multiple proportions by specific antisera. Within the same bacterial species, there may be considerable variation in the potency of filtrates obtained from cultures of various strains. In a given filtrate, the potency seems to parallel the lethal effect on rabbits.

The demonstration of exotoxins of a new type in a large number of unrelated organisms as the members of the *coli*-typhoid-dysentery group, the meningococcus, the Pneumococcus Type III, the *Streptococcus haemolyticus-scarlatinae* (Shwartzman (12)), the gonococcus (Shwartzman (14)), the *Bacillus influenzae* (*Haemophilus influenzae*) (Frisch (15)), the *Bacillus pertussis* (Gross (16)), and the tubercle bacillus (Bieling (17)) was of great importance.

It was deemed of interest to determine whether this type of bacterial toxin could be converted into a toxoid state. With this aim in view, the following experiments were undertaken.

### *Methods and Materials*

These experiments were performed on rabbits which had been employed once within the previous week for the elicitation of the Shwartzman phenomenon, receiving for this purpose a variety of materials. Only those rabbits were selected in which the phenomenon had failed to occur. Unpublished experiments suggest that such rabbits may be more sensitive to the Shwartzman phenomenon than stock rabbits. Further experiments upon this point are now under way.

*Toxins.*—(a) Meningococcus "agar washings" toxic filtrates were prepared and titrated as described by Shwartzman (18, 19). Filtrate 1409 was prepared from meningococcus Strain 123, Group I; Filtrate 1675 from meningococcus Strain 383, Group II; Filtrate A52 was pooled from Filtrates 1233A, 1233B, 1233C, and 1233D, prepared from meningococcus Strains 44B Serum 8, 44B Serum 13, 44B Serum 15, and 44B Serum 16, respectively, all Group III (Shwartzman (20)). Meningococcus Filtrate 1675 was kept in the refrigerator at 6°C. from June 16, 1931, to Dec. 18, 1931 (time of retitration). The titers of the preparations are indicated in Table I.

(b) 1. 147 cc. of meningococcus "agar washings" toxic Filtrate 1409 plus 3 cc. of 20 per cent formalin to make a total volume of 150 cc. with a final concentration of 0.4 per cent formalin were placed in a ground glass-stoppered bottle, sealed

TABLE I  
*Titration of Polency of Skin-Preparatory and Reacting Factors of Filtrates Studied*

Group No.	Skin-preparatory factor (0.25 cc. of undiluted filtrate)	Dose per kw.*		No. of reacting units	No. of rabbits	No. of rabbits showing reactions	Titer in reacting units per cc.
1	mingo† T‡ 1:09	1 cc. mingo	T 1:09	diluted 1:1200	3	2	1400
2	" " 1:09	1 "	" 1:09	" 1:1600	3	0	
3	" " Toxoid 1:09A	1 "	" 1:09	" 1:22	3	2 (1 weak)	
4	" " T 1:09	1 "	" Toxoid 1:09A	" 1:22	3	1 (weak)	
5	" " Toxoid 1:09B	1 "	" T 1:09	" 1:11	3	0	
6	" " " 1:09B	1 "	" " 1:09	" 1:22	3	0	
7	" " T 1:09	1 "	" Toxoid 1:09B	" 1:11	3	1	17
8	" " " 1:09	1 "	" " 1:09B	" 1:22	3	0	
9	" " T A52	1 "	" T A52	" 1:500	3	1	750
10	" " " A52	1 "	" " A52	" 1:1000	3	0	
11	" " 1:09	1 "	" Toxoid A52	" 1:15	3	1	23
12	" " " 1:09	1 "	" " A52	" 1:30	3	0	
13	" " 1675 (July)	1 "	" T 1675	" 1:1100	3	1	1300
14	" " 1675 "	1 "	" " 1675	" 1:1500	3	0	
15	" " 1675 (Dec.)	1 "	" " 1675	" 1:100	3	1	150
16	" " 1675 "	1 "	" " 1675	" 1:200	3	0	

\* kw. = kilo of body weight.

† mingo = meningococcus.

‡ T = toxin.

§ ? = Toxoid 1:09A not titrated to the end-point.

*Titration of Meningococcus Skin-Preparatory and Reacting Factors*

It has been demonstrated by Shwartzman (13) that there exists a quantitative reciprocal relationship between the skin-preparatory and reacting factors of unrelated potent toxins. Therefore, with the use of meningococcus Toxin 1409 skin-preparatory factors it was possible to test meningococcus Toxoids 1409A and B for reacting factors and with meningococcus Toxin 1409 reacting factors to test meningococcus Toxoids 1409A and B for skin-preparatory factors. The results are summarized in Table I.

As is seen from Table I (Groups 1 and 2), meningococcus Toxin 1409 had a titer of 1400 reacting units. Meningococcus Toxoid 1409A used for preliminary experiments was not titrated to the end-point. However, it is seen from Table I (Groups 3 and 4), that there was a considerable reduction in skin-preparatory and reacting potency. Thus, of 3 rabbits which received 0.25 cc. of undiluted meningococcus Toxoid 1409A intradermally, and 24 hours later an intravenous injection of 64 reacting units of meningococcus Toxin 1409 per kilo of body weight, 1 rabbit developed a strong and 1 a weak reaction. Of 3 rabbits which received 0.25 cc. of undiluted meningococcus Toxin 1409 intradermally and 24 hours later an intravenous injection of 1 cc. of meningococcus Toxoid 1409A diluted 1:22 (a dilution corresponding to 64 reacting units of meningococcus Toxin 1409—the mother filtrate) per kilo of body weight, only 1 developed a positive reaction and that of moderate intensity. After an intravenous injection of 64 reacting units of meningococcus Toxin 1409 at least 2 of 3 rabbits show strongly positive reactions.

Meningococcus Toxoid 1409B had no skin-preparatory factors for 64 and 128 reacting units of meningococcus Toxin 1409 per kilo of body weight (Table I, Groups 5 and 6). Using meningococcus Toxin 1409 skin-preparatory factors, meningococcus Toxoid 1409B revealed only about 17 reacting units per cc. (Table I, Groups 7 and 8). Meningococcus Toxoid 1409B, therefore possessed no skin-preparatory factors and only 1.2 per cent of the reacting factors present in the mother filtrate.

Incidentally, it is of interest to note that following the intradermal injection of a toxoid preparation, there developed at the injected skin site an area of blanching 10 to 15 mm. in diameter surrounded by a

TABLE II  
*Titration of Antibody-Combining Capacity of Filtrates Studied*

Group No.	Preparation	Skin-preparatory factors	Titer of reacting factors in units per cc.	Intravenous injection: 1 cc. of following dilution per kw.	Sera used for neutralization in amounts per kw.	Results of neutralization titrations		
						CN*	IN†	NN‡
1	mngo T 1409	+§	1400	1:14	0.9 cc. Serum H251	100		
2	" " 1409	+	1400	1:10	+0.1 cc. Serum H230		140	
3	" " Toxoid 1409B	-	17	1:10	" "	1.7		
4	" " 1409B	-	17	1:7	" "		2.4	
5	" " 1409B	-	17	Undiluted	" "		17	
6	" " T 1675 (July)	+	1300	1:10	+0.9 cc. Serum H251	130		
7	" " 1675 "	+	1300	1:7.5	+0.1 cc. Serum H290			175
8	" " 1675 (Dec.)	+	1500	1:13.6	" "	11		
9	" " 1675 "	+	1500	1:10	" "			15

\* CN = consistent neutralization.

† IN = irregular neutralization.

‡ NN = no neutralization.

§ + = 0.25 cc. of undiluted filtrate was able to prepare the skin of a rabbit for the phenomenon.

|| - = no skin-preparatory potency.

purpuric zone 1 to 2 mm. in width. A similar observation has been made by Burnet (22) who suggested that spontaneous local desensitization might be taking place in the center of the skin sites injected with toxoid preparations. However, control injections of 0.25 cc. of 1 per cent formalin produce the same effect. Pituitrin (Klein (23)) and adrenalin have a similar action on the rabbit's skin. This circinate distribution of the purpuric reaction at the prepared skin site must therefore be considered as a non-specific manifestation of primary local toxicity of the injected substances.

A Besredka (24) disintegrate of a *B. dysenteriae* Flexner strain to which 0.2 per cent formalin had been added and which had been kept at 37°C. for 1 month was referred to by Burnet as toxoid. His toxoid preparation suffered a loss only in skin-preparatory, but not in reacting potency (no titrations to the end-point), as compared with the mother filtrate. Studies of the antibody-combining capacity or antigenicity are not mentioned.

#### *Titration of Antibody-Combining Capacity of Meningococcus Toxoids*

Neutralized mixtures of meningococcus Toxin 1409 and meningococcus Toxoid 1409B with antimeningococcus horse Serum H251 and auxiliary antibody Serum H230 were titrated as described by Schwartzman (21). The results are summarized in Table II (Groups 1 to 5).

As is seen from Group 1 of Table II, consistent neutralization was obtained with 100 units of meningococcus Toxin 1409 (1 cc. of a 1:14 dilution of meningococcus Toxin 1409). With the use of an equal amount of serum, consistent neutralization was obtained with only 1.7 units of meningococcus Toxoid 1409B (1 cc. of a 1:10 dilution of meningococcus Toxoid 1409B) (Table II, Group 3). The ratio of the antibody-combining capacity of meningococcus Toxoid 1409B to that of meningococcus Toxin 1409 is, therefore, that of 10:14, thus showing in the toxoid a loss of only 28.7 per cent of the antibody-combining capacity associated with a 98.8 per cent loss of toxicity as compared to the mother filtrate.

It remained to determine whether 1.7 reacting units of meningococcus Toxoid 1409B actually held in combination all of the neutralizing antibody in the serum used for the neutralization titrations (0.9 cc. of Serum H251 plus 0.1 cc. of Serum H230). If the mixture contained an excess, *i.e.* unbound antibody, then consistent neutralization

December, the toxin retained but 11.5 per cent of the July reacting titer. In contrast with this, in December, it retained 73.5 per cent of the antibody-combining capacity present in July. The relative preservation of the antibody-combining capacity, despite the decrease in titer of reacting units which takes place in meningococcus toxin on standing, resembles closely, therefore, the transformation of diphtheria toxin into toxoid under similar conditions.

### *Lethal Effect of Meningococcus Toxoid*

An intravenous injection of an "agar washings" toxic filtrate produces a considerable mortality in rabbits. There appears to be a definite parallelism between the lethal effect and the titer of reacting

TABLE III  
*Lethal Effect of Filtrates Studied*

Group No.	Intravenous injection per kwt.	No. of reacting units	No. of rabbits	Result
1	1 cc. mngo T A52 diluted 1:5	150	3	1 died after 2 hrs. 2 survived
2	1 " " Toxoid A52 undiluted	23	3	All survived
3	2 " " " A52 "	46	3	" "

units of a given filtrate (Shwartzman (13)). It was of interest, therefore, to determine whether the decrease brought about in the titer of reacting units in meningococcus toxoid preparations, was associated with a parallel decrease in lethal effect.

From Table I (Groups 9 and 10), it can be seen that meningococcus Toxin A52 possessed a titer of 750 reacting units. In contrast to this meningococcus Toxoid A52 possessed a titer of only about 23 reacting units (Table I, Groups 11 and 12). The lethal effect of both of these filtrates was studied. The results are summarized in Table III.

Of 3 rabbits which received intravenously 1 cc. of meningococcus Toxin A52 diluted 1:5 (150 units) per kilo of body weight, 1 died 2 hours after the injection, and the others survived (Table III, Group 1). Two groups, each of 3 rabbits, received intravenous injections of 1 cc. and 2 cc., respectively, of undiluted meningococcus Toxoid A52 per kilo of body weight; all survived (Table III, Groups 2 and 3).

should still be obtained after the addition of a small amount of toxin to the mixture. Accordingly the following experiment was performed

Each of 4 rabbits received an intradermal injection of 0.25 cc. of undiluted meningococcus Toxin 1409 followed 24 hours later by an intravenous injection of a mixture of 1 cc. of meningococcus Toxoid 1409B diluted 1:10 (1.7 reacting units) plus 0.9 cc. of Serum H251 plus 0.1 cc. of Serum H230 plus 0.5 cc. of meningococcus Toxin 1409 diluted 1:70 (10 reacting units) per kilo of body weight. Reactions were elicited in 3 of the 4 rabbits (no neutralization).

This demonstrates conclusively that 1.7 reacting units of meningococcus Toxoid 1409B actually combined with all of the neutralizing antibody in 0.9 cc. of Serum H251 plus 0.1 cc. of Serum H230.

### *Spontaneous Meningococcus Toxoid Formation*

The titer of reacting units of a bacterial filtrate potent in eliciting the phenomenon often diminishes on standing (Shwartzman (13)). It was observed by Ehrlich (1) that, on standing, diphtheria toxin often suffered a decrease in the titer of direct toxicity whilst the antitoxin-combining power remained essentially unaltered. It was, therefore, of interest to determine whether the decrease in the titer of reacting units of a bacterial filtrate which occurred on standing was associated with a diminution of the antibody-combining capacity.

For this purpose the following was done.

The preparation of meningococcus "agar washings" toxic Filtrate 1675 was completed on June 16, 1931. In the period, July 15 to 17, 1931, the titer of reacting units and the antibody-combining capacity were determined. These determinations were performed on stock rabbits. Antimeningococcus horse Serum H251 and anticoli horse Serum H290 were used for the neutralization experiments. In the period Dec. 18 to 23, 1931, these determinations were repeated on used rabbits. The results are summarized in Table I (Groups 13 to 16) and Table II (Groups 6 to 9).

As is seen from Table I, despite the possibly increased sensitivity of used rabbits, the titer of reacting units dropped from 1300 to 150 units. However, the same amount of serum (0.9 cc. of Serum H251 plus 0.1 cc. of Serum H290) which consistently neutralized 1 cc. of a 1:10 dilution of meningococcus Toxin 1675 in July was necessary for consistent neutralization of 1 cc. of a 1:13.6 dilution of meningococcus Toxin 1675 in December (Table II, Groups 6 to 9). Therefore, in

TABLE IV  
*Agglutination Titration of Meningococcus Toxoid 1409B Antisera*

Serum employed	Antigen employed for preparation of serum	Serum dilution									
		1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2580	1:5120
Serum Rabbit 9-50	Meningococcus Toxoid 1409B	++	+++	+++	++	++	++	0	0	0	0
Serum Rabbit 9-40	"	+++	+++	+++	+++	+++	++	++	+	±	0
Serum Rabbit 4-10	"	+	+	+++	+++	+++	+++	++	0	0	0
Serum Rabbit 3-32	Group 1 meningococcus vaccine	++++	++++	±*	++++	+	±	0	0	0	0

++++ = complete agglutination.

+++ = almost complete agglutination.

++ = partial agglutination.

+ = slight agglutination.

0 = no agglutination.

\* Interzone(?).



It can be stated that death following an intravenous injection of a meningococcus filtrate generally occurs within 4 hours. In these experiments, the surviving rabbits were observed for 3 days after the injection and showed no signs of intoxication.

Occasionally a death occurs after an intravenous injection of 10 to 25 units per kilo of body weight. An injection of from 50 to 100 units is frequently followed by death. It appears that an injection of from 100 to 200 units is regularly associated with mortality, the percentage being proportional to the dosage. From Table III (Group 1), it can be seen that an injection of 150 units of meningococcus Toxin A52 was followed by the death of 1 of 3 rabbits. From Groups 2 and 3, it can be seen that the injection of 1 and 2 cc. of meningococcus Toxoid A52 representing the volume of 750 and 1500 units of the mother filtrate (meningococcus Toxin A52) per kilo of body weight, was tolerated perfectly well by the 6 animals injected. This demonstrates a striking diminution in the lethal effect of the toxoid as compared to the mother filtrate.

#### *Antigenicity of Meningococcus Toxoid*

It remained to ascertain the antigenicity of a meningococcus toxoid preparation; e.g., meningococcus Toxoid 1409B. Lowenstein (6) had noted that the antigenicity of a tetanus toxoid was directly proportional to its antitoxin-combining capacity. In view of the fact that meningococcus Toxoid 1409B possessed 71.3 per cent of the antibody-combining capacity of the mother filtrate (meningococcus Toxin 1409), it was expected that the toxoid would still be antigenic.

6 rabbits were immunized, each by intradermal injections of 0.5 cc. of undiluted meningococcus Toxoid 1409B on Jan. 30, Feb. 5, 10, 17, and 24, and by intravenous injection on Feb. 6, 11, 18, and 25 of 1 cc. of meningococcus Toxoid 1409B diluted 1:20, 1:10, 1:5, and undiluted, respectively, per kilo of body weight. 3 of the rabbits survived until Feb. 29, when they were bled. A standard suspension of a 24 hour culture of meningococcus Strain 123, Group 1 (the strain which furnished meningococcus Toxin 1409), was agglutinated with the sera of these animals. The results are set down in Table IV. For comparison, the same suspension of meningococci was agglutinated with Serum R332, a stock, Group 1, meningococcus, rabbit agglutinating serum.

As can be seen from Table IV, the agglutinating titers of the sera produced by immunization with meningococcus Toxoid 1409B varied

it is suggested that they be employed in the production of therapeutic sera.

As yet unpublished experiments suggest that serum from certain human beings may possess the capacity to neutralize meningococcus reacting units. Since meningococcus toxoid is antigenic and has a very low titer in reacting units (relatively non-toxic), it may possibly be of use in the active immunization of man against meningococcus infections.

It is still common practice to evaluate therapeutic meningococcus sera by their agglutinin titers. However, this criterion of evaluation reveals a lack of correlation between the agglutinin titer and the therapeutic efficacy of a serum. Recently, Schwartzman (20, 25) has suggested that therapeutic meningococcus sera be graded by their capacity to neutralize meningococcus reacting factors. However, the demonstration of toxoid formation should be taken into consideration *inasmuch as, in filtrates with different degrees of toxoid formation, the same number of reacting units may represent varying antibody-combining capacities.*

It would seem advisable to keep as a standard an immune serum which had been titrated against freshly prepared meningococcus toxin, in which only insignificant, if any, toxoid formation had occurred. Other sera could then be compared to this by their capacity to neutralize any given meningococcus filtrate.

#### SUMMARY AND CONCLUSIONS

Formalin induces a considerable change in meningococcus culture filtrates. This consists of a marked decrease in toxicity as concerns both the Schwartzman phenomenon and the lethal effect, with relative preservation of the antibody-combining capacity and antigenicity.

A similar modification occurs spontaneously in meningococcus culture filtrates on standing.

Inasmuch as these changes parallel those occurring in the conversion of diphtheria toxin into toxoid, it is justifiable to consider such altered meningococcus toxin as meningococcus toxoid.

I am indebted to Dr. Gregory Schwartzman for many kindnesses and invaluable assistance.

from 320 to 1280, whereas the control serum had a titer of only 160. Sera obtained from trial bleedings of the rabbits performed January 28, 1932, were free of spontaneous agglutinins for meningococci. The sera contained no precipitins either for meningococcus Toxin 1409 or meningococcus Toxoid 1409B. The sera were unable to neutralize meningococcus Toxin 1409 reacting factors (1 cc. of serum mixed with 3 units of toxin). However, rabbits do not readily form precipitins for bacterial filtrates nor are they good antitoxin producers. Nevertheless, further studies are under way with the aim of producing precipitating and antitoxic rabbit sera.

#### DISCUSSION

The experiments cited demonstrate that meningococcus culture filtrates treated with formalin and heat can be modified similarly to diphtheria toxin. The toxicity, as measured by the titer of reacting units, and the lethal effect are markedly decreased. In contrast, the antigenicity and the antibody-combining capacity are essentially unimpaired.

Furthermore, no comparable loss in antibody-combining capacity is associated with the decrease in toxicity occurring on standing. Ramon (4) by precipitation tests has found that diphtheria toxoid flocculates with diphtheria antitoxin to the same titer as the mother filtrate (toxin).

It was the detoxifying alteration of the sort undergone by diphtheria toxin that led Ehrlich to the description of diphtheria toxoid. Since meningococcus culture filtrates can spontaneously or artificially be modified in a similar manner, it appears justifiable to consider such a modified meningococcus culture filtrate as meningococcus toxoid.

As indicated in the introductory section, all of the classical toxins can be converted into toxoid states. The production of a meningococcus toxoid is further evidence of the similarity in properties between the bacterial exotoxins studied by Shwartzman and the classical bacterial exotoxins, *e.g.*, diphtheria and tetanus.

The demonstration of a meningococcus toxoid has a considerable practical importance. It is common experience that animals used for the production of therapeutic sera eventually succumb to the toxic effects of the injected antigens. Since toxoids show little lethal effect,

# INFECTIOUS MYXOMATOSIS (SANARELLI) IN PREGNANT RABBITS

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PLATES 32 AND 33

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Viruses exhibit an intimate type of parasitism. This phenomenon is emphasized by the fact that many of the active agents multiply only in one species of host and only in certain tissues of that host. In the course of some experiments with infectious myxomatosis it was noticed that the clinical picture and morbid anatomy of the disease in pregnant rabbits are different from those in non-gravid animals. These differences indicate a line of approach to a better understanding of the host-virus complex and are sufficiently striking to be recorded.

Many investigators have observed that the course of certain tumors is influenced by pregnancy. Inasmuch as the reports and views regarding this matter are contradictory, some of the more prominent ones are cited. Morau (1), Ehrlich (2), Uhlenhuth and Weidanz (3) reported that pregnancy retards the growth of transplantable tumors in mice. Cuénot and Mercier (4), however, observed an acceleration of growth of similar tumors, while Albrecht and Hecht (5) stated that pregnancy has no effect on such neoplasms. Askanazy (6), Jentzler (7), and Herzog (8) reported that the growth of tumors in rats was accelerated by pregnancy. Kross (9), however, claimed that pregnancy has no effect on the rate of growth of neoplasms in rats.

Rous (10) in studying the effect of pregnancy on embryonic transplants, which he had shown to behave in many respects like transplantable tumors, reported the following experiments. Several loops of the uterus of a pregnant mouse were removed. A small fragment from one of the embryos was injected beneath the skin of the mother. This piece of tissue became vascularized but did not grow until the remaining part of the uterus was emptied. In other experiments he found that embryonic transplants grew equally well in non-gravid mice and in pregnant mice but that the transplants became differentiated into a larger number of tissues in the latter group of animals. Fischera (11) made similar observations concerning embryonic transplants in rats. Shattock, Seligmann, and Dudgeon (12), however, reported that gestation had no effect on the growth of transplanted fetal cartilage.

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mammary tissue has an increased blood supply during pregnancy which might affect the local lesion and the dissemination of the virus. The virus was diluted with Locke's solution in multiples of 10 up to  $1.0 \times 10^{-6}$ , 0.25 cc. of the different dilutions being injected as indicated below.

*Fixation and Stains.*—Tissues used for histological study were removed from the animals within an hour after death except in the instances where it appeared that a rabbit would not live through the night. Under such conditions the animal was killed and necropsy was performed immediately. Tissues from the heart, lungs, spleen, liver, adrenals, kidney, ovaries, intestines, bone marrow (shaft of the femur), skin, lymph nodes, and brain were fixed in Zenker's fluid (5 per cent acetic acid) and in formalin (10 per cent or 3.7 per cent formaldehyde gas) and stained with hematoxylin and eosin, with Mallory's phosphotungstic acid hematoxylin, and according to the Giemsa method. Sections of the liver were stained with Sudan III for fat.

#### EXPERIMENTAL

Experiments were designed to ascertain whether pregnancy altered the resistance of rabbits to the virus. Hence some of the animals were given relatively small amounts of the active agent.

*Experiment 1.*—Each of 3 pregnant and 2 non-pregnant female rabbits received intradermal inoculations, 0.25 cc., of dilutions of the same virus emulsion ranging from  $1.0 \times 10^{-2}$  to  $1.0 \times 10^{-6}$ .

*Experiment 2.*—Each of 6 pregnant rabbits and 1 non-pregnant female rabbit were given 3 intradermal injections, 0.25 cc., of dilutions of the virus emulsion ranging from  $10^{-4}$  to  $10^{-6}$ .

*Experiment 3.*—Each of 4 pregnant and 8 non-pregnant female rabbits received 6 intradermal injections, 0.25 cc., of dilutions of the virus emulsion ranging from  $10^{-1}$  to  $10^{-6}$ .

In the 3 experiments, 13 pregnant and 11 non-pregnant female rabbits were inoculated with the virus of infectious myxomatosis and the resulting clinical picture and morbid anatomy were observed. Inasmuch as the results of the experiments were similar, they will not be described separately.

*Clinical Picture in Non-Pregnant Rabbits.*—On the 3rd day slightly elevated skin lesions appear at the points of inoculation. These lesions increase in size and on the 5th day secondary lesions in the skin are usually visible. The primary lesions at this time become hemorrhagic and are capped by small vesicles. The subcutaneous tissues of the eyelids, ears, and genitalia as a rule show involvement on the 6th day and by the 9th the eyes are closed. On the day of the disease on which the animals die, usually the 9th after inoculation, they are dyspneic.

The effect of pregnancy on spontaneous tumors in animals has not been investigated to any extent. Slye (13) stated that neoplasms occur less frequently in gestating mice than in virgin mice. Krotkina (14) observed that in a rabbit with a tar cancer the neoplasm became smaller during each pregnancy. Rous (10) observed that the growth of a spontaneous tumor in one mouse was accelerated during pregnancy.

The reports concerning neoplasm in pregnant women are with a few exceptions in accord. Bainbridge (15) in an excellent review of the literature concluded that pregnancy exerts a stimulating and hence malign influence upon coexistent cancer. This he believes to be true not only of cancer of the breast and uterus where an increase in the blood supply might account for the accelerated growth but also of neoplasms in other parts of the body with the possible exceptions of certain epitheliomas.

Taliaferro (16) and his associates found that during the latter stages of pregnancy the resistance of rats to *Trypanosoma lewisi* is lowered. No other reports of experimental work dealing with the effect of pregnancy on diseases due to microorganisms have been encountered.

The mortality in acute infections is generally considered to be increased by pregnancy. This change is attributed by Williams (17) to the extra strain of the abortion which accompanies the infection. Weintraub (18) pointed out in his review of the literature on influenza that women may not die because they abort but abort because they are dying. Many authors have claimed that the susceptibility to influenza is increased by pregnancy. Weintraub (18) is of the opinion that this may be only apparent since influenza is most common in early adult life, the period during which women are most frequently pregnant. It has been maintained that the incidence of acute infections among pregnant women is lower than among the non-pregnant. With the exception of scarlet fever (19) there appears little evidence to support this hypothesis.

### *Methods and Materials*

*Virus.*—The virus employed in these experiments is the strain used by Rivers (20). Its virulence may have been somewhat enhanced by numerous passages through animals. The virus was prepared by dissecting the myxomatous material, occurring at the point of an intradermal inoculation of the virus, free from skin and muscle. The infectious tissue was then ground thoroughly with alundum and diluted with sufficient distilled water to make a 10 per cent suspension. The virus was found to be active in such suspensions diluted  $1.0 \times 10^{-4}$  and  $1.0 \times 10^{-5}$ .

*Animals.*—Pregnant and non-pregnant female rabbits from the same source were used. The gestation period of the gravid animals had proceeded for about 20 days—the normal gestation period is about 30 days.

*Inoculation.*—The rabbits were inoculated intradermally. The points chosen for inoculation were well up on the rabbit's side in order that the mammary glands would not be involved. This precaution was considered necessary because the

*Morbid Anatomy of the Pregnant Rabbits. Liver.*—All the rabbits, except one, shown an acidophilic necrosis of the hepatic cells around the central vein (Fig. 3). In some instances the destruction involves practically the entire lobule but there is never any cellular infiltration. In the periportal region of one liver there are a number of stellate elements that resemble the myxomatous cells seen in the spleen. Microscopic examination of the livers from 2 normal rabbits which had just given birth to young reveals that the hepatic cells are filled with fat but are not necrotic.

*Skin.*—The lesions are not elevated above the surrounding tissue as much as are those in the non-pregnant animals. Histological preparations reveal lesions similar to those observed in the non-pregnant group except that there is less fibrin and the muscle layer appears to be less severely damaged.

*Lungs.*—All the pregnant animals have secondary lesions in the lungs similar to those described in the non-pregnant animals with the exception that they are much more extensive (Fig. 4). One lung showed a generalized infiltration of the interstitial tissue with large mononuclear cells.

*Spleen.*—All the spleens are increased in size and the structure is greatly distorted (Fig. 2). Practically no lymphocytes are seen and the Malpighian corpuscles are represented by nests of large stellate cells. Many small foci of necrosis are seen. In the sinuses of the splenic pulp are large amounts of fibrin. The endothelium of the blood vessel is swollen and granular. Macrophages have phagocytized a number of lymphocytes. A few polymorphonuclear cells and some lymphocytes with pyknotic nuclei are seen, but the majority of the cells in the splenic pulp are large stellate elements similar to those seen in the Malpighian corpuscles. Throughout the spleen there is an increase in the number of fibroblasts.

*Lymph Nodes.*—The amount of endothelial damage and hemorrhage and the number of fibroblasts seem to be greater in the lymph nodes of the pregnant rabbits than in similar structures of the non-pregnant animals.

*Ovary and Uterus.*—In the muscular layer of the pregnant uteri are seen a few stellate cells resembling the myxomatous cells in the spleen. Around and in the Graafian follicles of the ovaries similar cells are found.

*Heart, Adrenals, Kidney, Intestines, Bone Marrow, and Brain.*—No significant changes are present in these organs.

From the results of the experiments described above it is obvious that there are differences in the phenomena occurring in pregnant and non-pregnant rabbits infected with the virus of infectious myxomatosis. In pregnant rabbits the lesions at the point of inoculation are less elevated and the secondary lesions of the skin and involvement of the subcutaneous tissues of the ears are either entirely absent or much smaller than are those observed in the control group. More extensive lesions in the spleen and the presence of secondary lesions in the lungs of all the pregnant animals contrast sharply with the involvement of only



*Clinical Picture in Pregnant Rabbits.*—The course of the disease in the pregnant animals is similar to that described above with the following exceptions. The skin lesions are less elevated. Secondary lesions of the skin and involvement of the subcutaneous tissues of the ears are frequently absent or small. Approximately half of the animals abort on the 8th or 9th day of the disease. The remainder carry the young until death which usually occurs on the 9th day. The pregnant and non-pregnant animals responded in like manner to the various dilutions of the virus.

*Morbid Anatomy of Non-Pregnant Rabbits.*<sup>1</sup> *Liver.*—Small areas of focal necrosis are seen in the liver but such lesions are not uncommon in "normal" rabbits. In 2 animals a few necrotic cells are seen around the central vein.

*Skin.*—The lesions are slightly elevated, ovoid, firm, purplish masses that extend through the superficial muscles into the deeper fascial layer. A cross-section reveals a myxomatous-like tissue which contains numerous small hemorrhagic areas and dilated blood vessels. Histological preparations reveal hyperplasia, necrosis, and vesiculation of the epithelium. The cytoplasmic inclusions described by Rivers (20) are seen. Beneath the epithelium are some degenerating polymorphonuclear cells. Extending throughout the subcutaneous tissue are numerous small strands of fibrin. The superficial muscle layer is atrophied and in places necrotic. Around the hair follicles, glands, and blood vessels are a number of proliferating stellate cells, characteristic of the disease.

*Lungs.*—Only 4 animals in this group have secondary myxomatous lesions in the lungs. The lesions first appear in the lymphoid tissue around the bronchi and are frequently infiltrated with polymorphonuclear leucocytes. From this point of origin the pathological process extends around the blood vessels and into the interstitial tissue. The epithelial cells of the bronchi in close proximity to the myxomatous tissue are hyperplastic.

*Spleen.*—In only a few instances are the spleens enlarged. Myxomatous secondary lesions, however, are found in the Malpighian corpuscles of all the rabbits (Fig. 1). In most of the animals only a few follicles are involved while in others only a few normal lymphoid cells remain.

*Lymph Nodes.*—The architecture of the lymph nodes draining the region of inoculation is destroyed. The lymphoid cells are replaced by a large number of stellate elements among which are scattered numerous red blood cells, some fibroblasts, and fibrin. The endothelial cells lining the blood vessels are swollen and granular. In places such changes are sufficiently extensive to cause a loss in the continuity of the vessel wall.

*Heart, Adrenals, Kidney, Intestines, Bone Marrow, Brain, Ovary, and Uterus.*—No significant changes are found in these organs.

<sup>1</sup> The findings in the non-pregnant group are in agreement with the observations of Rivers (20) and Stewart (21), and for a more detailed description of the pathology the reader is referred to their papers.

to the reaction of the liver of the pregnant rabbit and the pregnant woman. Acute yellow atrophy occurs more frequently among pregnant than among non-pregnant women. The amount of arsenic that a non-pregnant woman can take with impunity results in an extensive central necrosis of the liver when given to a pregnant woman. The lesion is similar to that seen in the gravid rabbit with myxoma. Such observations indicate that the resistance of the hepatic cells to toxic substances is lowered during pregnancy. A pregnant woman with influenza is much more likely to have pneumonia than is a non-pregnant one (18). In like manner the pregnant rabbit has numerous secondary lesions of the myxoma in the lungs. The decrease in the resistance of the spleen to infectious myxoma resembles the lowered resistance of the spleen of pregnant women to leukemia (22).

#### SUMMARY

Pregnancy in rabbits alters the reactivity of the tissues to the virus of infectious myxomatosis. The livers of pregnant animals with the myxoma have a central acidophilic necrosis. Secondary lesions in the lungs are much more numerous and larger in the pregnant than in the non-gravid animals. In like manner the lesions in the spleen are more extensive in the pregnant rabbit. On the other hand the skin lesions of the pregnant animal are decreased in size.

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a few of the Malpighian corpuscles of the spleen and only an occasional secondary lesion in the lungs of the non-pregnant group. A necrosis of the hepatic cells around the central vein occurred in all except one of the pregnant animals, while only a few small areas of central necrosis were seen in 2 of the non-pregnant rabbits. In spite of these differences the animals in the 2 groups lived the same number of days after inoculation.

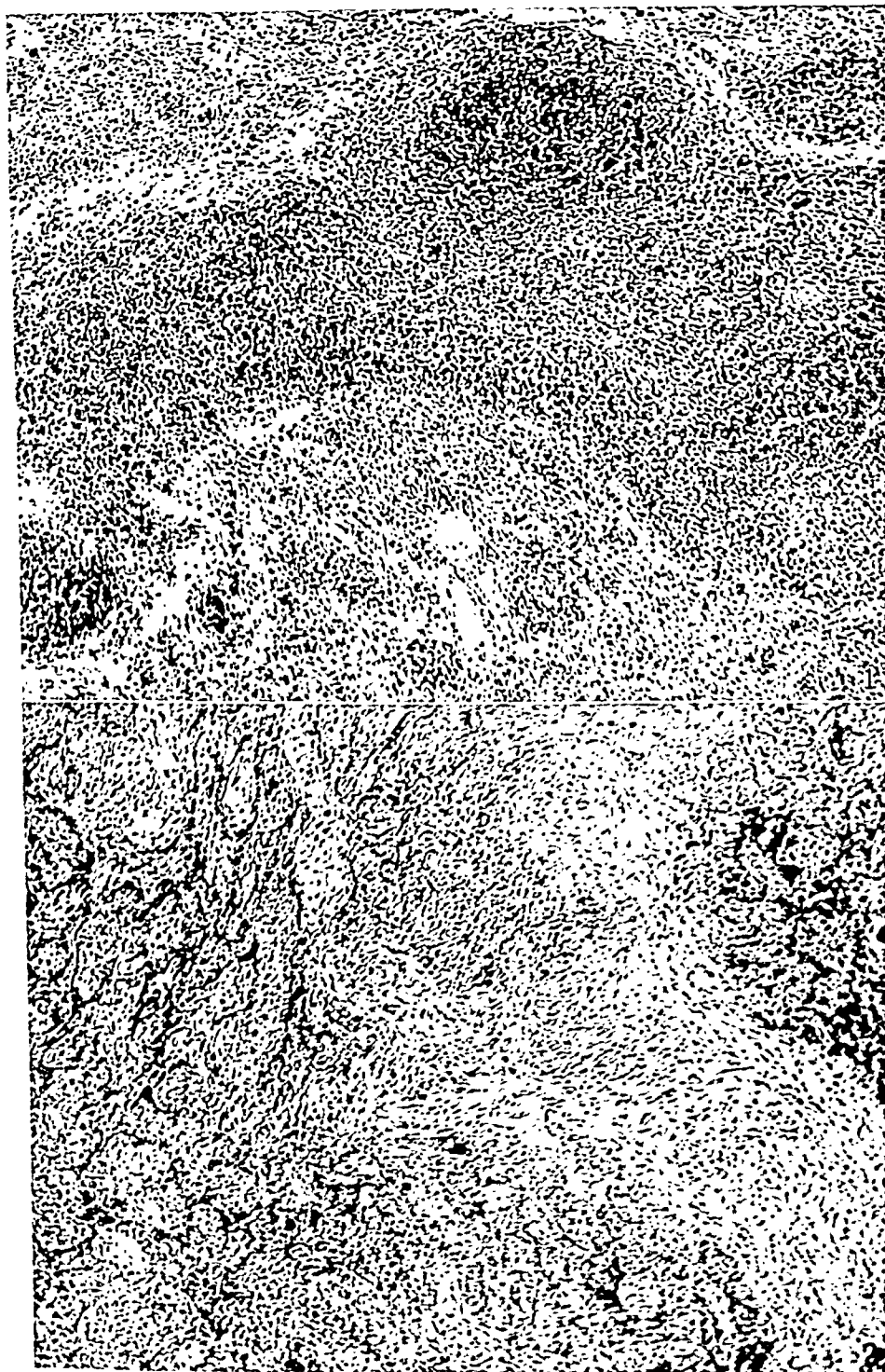
#### DISCUSSION

Pregnancy apparently does not change the general resistance of the rabbit to infectious myxomatosis because all the animals, both the pregnant and the non-pregnant, lived the same number of days following inoculation of the virus. The resistance, however, of the various organs in the pregnant animals appears to have been altered—that of the lungs, spleen, and liver is lowered while that of the skin is elevated. This interpretation is of course open to question, as one might maintain that the spleen of the pregnant animal, for example, is more resistant since it evidenced a greater degree of reaction.

In passing one should note that a similar decrease in the resistance of the internal organs of pregnant women to invasion by malignant neoplasms is generally found. The apparent increase in resistance of the skin of pregnant women to certain epitheliomas (15), however, is also of interest in connection with the increased resistance of the skin and subcutaneous tissue of the pregnant animal to infectious myxomatosis.

The conflicting reports of the results of investigation on the influence of pregnancy on the various experimental tumors in animals make a comparison of them with the findings reported here of little value. Furthermore, only the last third of pregnancy was investigated in the present experiments whereas the tumor experiments of other workers extended throughout the whole reproductive cycle, gestation, lactation, and resting phase. It is important to keep this fact in mind, because 2 rabbits inoculated in the earlier period of pregnancy reacted like non-pregnant animals.

The results of this investigation are more comparable to the observations on the effect that acute diseases have on pregnant women, and it is here that we find the most striking similarity, particularly in regard



Photographed by Louis Schmidt

(Sprunt. Infectious myxomatosis in pregnant rabbits)

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## EXPLANATION OF PLATES

## PLATE 32

FIG. 1. Histological preparation of a spleen from a non-pregnant rabbit with infectious myxomatosis showing only a slight myxomatous change. Hematoxylin and eosin.  $\times 90$ .

FIG. 2. Histological preparation of a spleen from a pregnant rabbit with infectious myxomatosis showing marked distortion and obliteration of splenic architecture. Hematoxylin and eosin.  $\times 90$ .

## PLATE 33

FIG. 3. Histological preparation of the liver of a pregnant rabbit with infectious myxomatosis showing a central necrosis of the hepatic cells. Hematoxylin and eosin.  $\times 47$ .

FIG. 4. Histological separation of a lung of a pregnant rabbit with infectious myxomatosis showing secondary lesions around a bronchiole and a hyperplasia of the bronchial epithelium. Hematoxylin and eosin.  $\times 47$ .



Photographed by Louis Schmitt

(5) cent. Infectious myxomatosis in pregnant rabbit.



STUDIES ON THE RELATIONSHIP OF STREPTOCOCCUS  
HEMOLYTICUS TO THE RHEUMATIC PROCESSI. OBSERVATIONS ON THE ECOLOGY OF HEMOLYTIC STREPTOCOCCUS IN  
RELATION TO THE EPIDEMIOLOGY OF RHEUMATIC FEVER

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To the student of rheumatic fever there are, in the natural history of this disease, few aspects of greater interest than the occurrence of explosive outbreaks. These epidemics are peculiarly significant in that they indicate that rheumatism is infectious in origin. The classical examples, described in Europe by Newsholme (1), by Hirsch (2) and by Andrieu (3), are well known; while the smaller outbursts of rheumatism in urban populations, in certain families, at congested military camps, in convalescent hospitals and in institutions, although rather obscure, constitute, nevertheless, an even more interesting chapter in the history of this disease. Although the clinical data of these outbreaks have been carefully collected, bacteriological studies of these previous waves of rheumatism have been lacking. Quite recently, however, bacteriological observations have been made by workers in England (4, 5) and in America (6), and their findings have indicated the importance of the association of hemolytic streptococcus with these explosive outbreaks. The present series of studies is concerned primarily with two aspects of the problem: first, the relationship of hemolytic streptococcus throat infection to the development of rheumatic fever; second, the association between recrudescences in rheumatic patients and preceding infection with *Streptococcus hemolyticus*. The bacteriological findings in this laboratory and those of other workers, together with necessary clinical data, are here recorded to emphasize this ecological relationship.

1. *The Correlation between the Ecology of Hemolytic Streptococcus and the Geographical Distribution of Rheumatic Fever.*—For many years it has been recognized that rheumatism is especially severe and





From these data it is observed that São Paulo is not distant from Santos and Rio de Janeiro; that it is not so densely populated as the latter; that it experiences a comparable humidity (less than Santos) but that its minimum and average monthly temperature is much lower than that of the other cities. This air temperature of 1.7°C. absolute minimum and monthly average 13.9–20.6° is comparable to the temperature in New York City during the spring months, when the incidence of rheumatic fever is high.

Physicians in Brazil have established the fact that scarlet fever appears in the mountainous regions of São Paulo and is extremely rare in the seashore city of Santos only a few miles away. The mortality statistics, 1921 to 1927, record deaths from scarlet fever as follows: São Paulo, 63; Santos, 1; Rio de Janeiro, 9. This was in proportion to the findings between 1914 and 1920: São Paulo, 286; Santos, 3; Rio de Janeiro, 15 recognized deaths from scarlet fever. The morbidity statistics, though less accurate, have been investigated with great care and show a similar wide difference. In the coast cities, the morbidity among school children was about 0.6 per cent; while in São Paulo it was 5.2 per cent in 834 children and 6.3 per cent among 157 adults. Each year in São Paulo during the cold weather, two outbreaks of scarlet fever were experienced with the peaks in May and September.

These epidemiological data collected over a period of years indicate the seasonal incidence of a streptococcus infection of the upper respiratory tract. In the environment with variable atmospheric conditions, scarlet fever was prevalent during the periods of cold weather. In the environment with a warm climate and minimal variation in temperature, little scarlet fever occurred.

To study the influence of climate on the flora of the upper respiratory tract, contemporaneous studies were conducted in New York and in the tropics during 1929 and 1930. The findings (6)<sup>2</sup> demonstrated that the bacterial life in the constantly hot tropics<sup>3</sup> and the pharyngeal flora of urban inhabitants in the tropical island of Puerto Rico are almost constant from month to month. The normal flora consisted mostly of non-hemolytic streptococcus and Gram-negative cocci. Hemolytic streptococcus appeared in the normal throat rarely and only in small numbers. Milam and Smillie (8) in their extensive bacteriological study of "colds" on an isolated tropical island (St. Johns, United States Virgin Islands, West Indies), also arrived at the con-

<sup>2</sup> Coburn (6), p. 221.

<sup>3</sup> This work was done in association with Dr. P. Morales Ortero of San Juan, Puerto Rico.

frequent in the north temperate zone. On the other hand, recent observations during the course of the present study (6),<sup>1</sup> and those of other workers have indicated the mildness of clinical manifestations and the relative rarity of the anatomical lesions of rheumatism in certain tropical countries. For this dissimilarity of incidence, an explanation has long been advanced that cold and dampness are important underlying factors in the pathogenesis of rheumatic fever. That humidity, dampness and cold may aggravate the symptoms of an individual who has had rheumatism and experienced tissue changes, has been repeatedly observed. However, during this study the direct influence of climate appeared to be of minor importance in initiating activity of the rheumatic process. There seemed to be a close and significant relationship between climate and the respiratory tract infections associated with rheumatic disease.

The influence of climate on a streptococcus disease is evident from certain aspects of the geographical distribution of scarlet fever. Scarletina is almost unknown in the tropical island of Puerto Rico; yet that susceptibility exists is demonstrated by the fact that Puerto Ricans contract scarlet fever after arrival in New York City. A more striking example of the influence of climate on the incidence of this hemolytic streptococcus infection exists in Brazil (7):

There, two cities of considerable population flourish only a 2 hours' railroad journey apart. São Paulo is in the mountains at an altitude of 800 m. and Santos, its port, is on the coast at sea level. The geographic and meteorological data are as follows:

	São Paulo	Santos	Rio de Janeiro
Latitude.....	23°32'48" S.	23°56' S.	22°54' S.
Longitude.....	46°38'10"	46°13' WG.	43°7'6" WG.
Altitude.....	800 m. above sea level	Seacoast	Seacoast
Air temperature			
Absolute maximum, °C.....	35.0°	36.0°	35.0°
Absolute minimum, °C.....	1.7°	10.4°	13.4°
Monthly average.....	13.9-20.6°	19.3-26.5°	19.3-25.0°
Relative humidity monthly averages, <i>per cent.</i> .....	77-83	77-86	72.8-80
Population.....	984,139	139,918	1,729,999

<sup>1</sup> Coburn (6), p. 118.

From these data it is observed that São Paulo is not distant from Santos and Rio de Janeiro; that it is not so densely populated as the latter; that it experiences a comparable humidity (less than Santos) but that its minimum and average monthly temperature is much lower than that of the other cities. This air temperature of 1.7°C. absolute minimum and monthly average 13.9–20.6° is comparable to the temperature in New York City during the spring months, when the incidence of rheumatic fever is high.

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clusion that the basic nasopharyngeal flora in St. Johns is quite constant throughout all seasons and in all groups of people. No change occurred in the nasopharyngeal flora in a group of persons who developed "colds." Hemolytic streptococci were rarely found in the Virgin Islands study. Only 3 per cent of the people harbored *Streptococcus pyogenes* and when found, the organisms were few in number.

2. *The Correlation between the Seasonal Distribution of Hemolytic Streptococcus and Rheumatic Fever.*—A study of rheumatism in New York City shows a striking seasonal variation. Observations on hospital ward patients and on ambulatory rheumatic subjects have

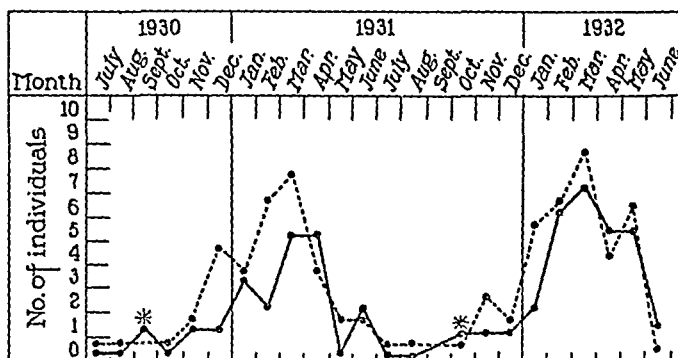


CHART 1. Seasonal incidence of hemolytic streptococcus pharyngitis and recrudescences of rheumatism amongst 165 ambulatory rheumatic subjects in New York City. The peaks occurred during the spring months, and were followed by a rapid rise in the incidence of recrudescences among ambulatory rheumatic subjects.

..... indicates hemolytic streptococcus pharyngitis.

———— indicates acute rheumatic fever.

\* Throat cultures not obtained during Sept., 1930 and 1931. Patients experienced preceding pharyngitis.

revealed each year a minimal number of acute attacks during the summer months, a moderate number in the fall and winter, and a sharp and marked increase in the spring.

In order to study the relationship of these fluctuations to incidence of *Streptococcus hemolyticus*, an investigation of the throat flora has been conducted in New York City over a period of years. These studies have demonstrated each year a wide seasonal variation in the

incidence of hemolytic streptococcus in the pharyngeal flora. In contrast to these variations, *Streptococcus viridans* has shown little change from season to season. No two years have been alike in the incidence of hemolytic streptococcus but certain trends have been similar. As an illustrative example, observations made in 1930-32 are presented in Chart 1.

That this phenomenon is not peculiar to the metropolitan population of New York is demonstrated by the interesting study conducted by the Ministry of Health in England (9).

This study was an intensive investigation of the throat flora in Manchester, England, during the period July, 1925, to Sept., 1927, and showed wide fluctuations in the incidence of hemolytic streptococcus. In 1925, mortality from respiratory disease was low in July and rose to a high peak in November. Hemolytic streptococcus, present in only 3.2 per cent of cultures in July, rose to a peak of 36.4 per cent in November and was persistently prevalent until Apr., 1926, when the incidence of this organism fell rapidly. In Aug., 1926, the incidence of and deaths from respiratory diseases reached a minimum, and hemolytic streptococcus entirely disappeared. The rapid rise in the frequency of respiratory disease in the fall of 1926 was accompanied by a slow steady rise in the incidence of hemolytic streptococcus. Again, with the peak of respiratory infection in January to March, hemolytic streptococcus reached a pinnacle, 17.8 per cent, subsiding gradually with the incidence of respiratory disease. In Sept., 1927, in contrast to the previous year, the incidence of this organism rose rapidly to 25 per cent, when respiratory infections were not prevalent. Whether this was followed by an early fall wave of respiratory disease is not known, for there this valuable study ended.

Corresponding to this seasonal high incidence of hemolytic streptococcus, the curve of incidence of acute rheumatism shows a similar form. In New York City the spring outburst of rheumatic fever has been preceded each year by a striking increase in incidence of hemolytic streptococcus in the throat flora. The positive correlation between the seasonal incidence of *Streptococcus hemolyticus* and the occurrence of acute rheumatism is a definite one.

3. *The Correlation between the Prevalence of Hemolytic Streptococcus and the Social Distribution of Rheumatic Fever.*—A marked difference in the incidence of rheumatic fever between the poor and the wealthy has long been emphasized. In an analysis (6)<sup>4</sup> of hospital patients in

<sup>4</sup> Coburn (6), p. 109.



private rooms and on the public wards at the Presbyterian Hospital in New York City, rheumatic fever appeared twenty times more frequently among the poorer classes than among those in comfortable circumstances. To investigate adequately the causes of this disproportion would be a tremendous undertaking. Preliminary studies of the throat flora have been conducted on five wealthy families and five pauper rheumatic families living only a few blocks apart on the Island of Manhattan. "Common colds" and the communicable diseases of childhood have been encountered in both groups with comparable frequency. The children of the wealthy were treated for these conditions with great care. The children of the poor enjoyed no advantages during convalescence, but instead lived on an inadequate diet, with poor sanitation, overcrowding, possibly a lack of sunshine and perhaps exposure to inclement weather. Comparable throat cultures obtained weekly in the two groups of children have during a period of 2 years demonstrated the occurrence of hemolytic streptococcus in 10 per cent of the cultures of the poor, and in only 1 per cent among the rich. In the first group, the initial appearance of active rheumatism has been observed in at least three instances; among the latter, there has been as yet no appearance of the rheumatic process. From this small group of ten families with 50 children, no final conclusions can be drawn. These preliminary findings suggest, however, that poverty and unhygienic living conditions favor both the activity of hemolytic streptococcus in the throat and the incidence of rheumatic fever.

4. *The Correlation between Infection with Hemolytic Streptococcus and the Localized Outbreaks of Rheumatic Fever.*—There is difficulty in dating the exact onset of the rheumatic state. In the absence of precise methods, it has not been possible to differentiate with certainty between the attack which represents the onset of the disease and the attack which represents a recrudescence. With this realization, a group of individuals apparently not yet stigmatized with rheumatism was selected that the influence of throat infection might be investigated.

In Sept., 1931, a class of 50 student nurses entered training at Presbyterian Hospital. The records of their histories and physical examinations obtained from their local doctors and the hospital physician indicated that they were

individuals in good health. Their living quarters were excellent, dry, sunny and at constant temperature. The diet was adequate. They were carefully watched to observe the course of events should they develop throat infections. During the fall months, two throat cultures were taken of each patient. In no instance was hemolytic streptococcus present in even moderate numbers, and from only three individuals were a few of these organisms recovered. Respiratory infections were inconspicuous in the fall; however, sore throats with hemolytic streptococcus in predominance on culture appeared among the student nurses during the winter and spring months in epidemic proportions.

*Monthly Incidence of Hemolytic Streptococcus Pharyngitis in a Group of Nurses*

	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.
No. of cases.....	—	—	—	6	2	8	8	2	2	—	—	—

Four of the group with throat infections developed rheumatic fever. Two types of inflamed throats were seen. In some, there was injection only; in others, there was also a sticky adherent slough. In these four individuals, whose throats had previously appeared normal, the onset of "sore throat" was accompanied by the advent of hemolytic streptococcus in almost pure culture. The manifestations of rheumatic fever were recognized after the subsidence of the local infection. In brief, the records are as follows:

*Individual 1.*—Contracted acute follicular tonsillitis on Mar. 6, 1931. Hemolytic streptococcus was recovered in almost pure culture from the throat (Strain RN1). During convalescence, she experienced epistaxis. On Apr. 27, rheumatism manifested itself with polyarthritis and mild carditis.

*Individual 2.*—Contracted acute follicular tonsillitis on Mar. 27, 1931. Hemolytic streptococcus was recovered in almost pure culture from the throat (Strain RN2). On Apr. 21, rheumatism manifested itself with joint pains and fever.

*Individual 3.*—Contracted acute pharyngitis on Feb. 27, 1931. Hemolytic streptococcus was the predominant organism in the throat culture (Strain RN3). On Mar. 10, rheumatism manifested itself with fever, muscle pains and mild polyarthritis.

*Individual 4.*—Contracted acute tonsillitis on Mar. 9, 1931. Hemolytic streptococcus was recovered in almost pure culture (Strain RN4). On Apr. 10, erythema marginatum appeared, and this was followed by other manifestations of rheumatic fever.

While under observation in the hospital environment, twenty-eight apparently normal, healthy individuals living in one building contracted acute hemolytic streptococcus throat infections in rapid succession. They were all of brief duration except for one case of

pneumonia. In four instances, however, after apparently complete recovery from the throat infection with *Streptococcus hemolyticus* rheumatic fever developed.

In England, observers have likewise reported epidemics of tonsillitis which were followed by the development of rheumatic fever.

In the fall of 1884, Haig-Brown (11) observed an epidemic of throat infection at the Charterhouse School. Of 345 cases of tonsillitis in previously healthy boys twenty-nine developed heart disease. In 1928 at the Halton Training School (10), there was an epidemic of 427 cases of tonsillitis, which was followed by forty-one cases of acute rheumatism. As pointed out by Glover, the tonsillitis preceded the rheumatism by 2 or 3 weeks. Bacteriological studies of these infections were not made; however, Glover and Griffith (4) recently described two epidemics of rheumatism in English boys' boarding schools in which 136 cases of "sore throat" were followed by fifteen cases of acute rheumatic fever.<sup>5</sup> In this epidemic, studies of the throat flora were made and showed a close association between the infection and hemolytic streptococcus. Moreover, in 1930 Bradley (12) encountered a similar epidemic of upper respiratory disease and emphasized the importance of hemolytic streptococcus.<sup>6</sup> The fact that in this school, free of rheumatic disease for more than 45 years, twenty-two individuals suffered from acute rheumatic fever following an epidemic of hemolytic streptococcus infections is significant.

The relationship of epidemics of tonsillitis to rheumatism has long been emphasized. These recent bacteriological studies have demonstrated a positive correlation between hemolytic streptococcus infection and localized outbreaks of rheumatic fever.

The four observations detailed above have been limited to the ecology of *Streptococcus hemolyticus* and its relationship to the initiation of the rheumatic process. In the following paragraphs the relationship of outbreaks of recrudescences among known rheumatic subjects to infection with hemolytic streptococcus is detailed.

*1. The Relationship of Outbreaks of Recrudescences in Isolated Rheumatic Colonies to the Appearance of Streptococcus Hemolyticus in the Throat Flora.*—In September, 1931, the author had under observation thirty girls with rheumatic heart disease at The Pelham

<sup>5</sup> Dr. F. Griffith has kindly sent two throat strains (G3 and G4) from this epidemic. These have been studied and are reported in Paper II, page 646.

<sup>6</sup> Dr. F. Griffith has kindly sent two throat strains (G1 and G2), the latter the Beatty culture. They have been studied and are reported in Paper II, page 649.

Home.<sup>7</sup> Of these only two were physically unable to attend school. There was in no instance definite clinical manifestation of an active rheumatic process. The tonsils had been removed in all except three individuals, and there was no indication of throat infection in any of the group. During the period of observation, the diet was adequate and liberal. All of the children gained weight. The Home was kept dry and heated at constant temperature during the school year. The only change during this period of observation was the discharge of five girls at the end of the first school term in March, and the admission of new patients in their places. A record of each individual's symptoms was charged daily, and each girl received a weekly physical examination.

The first rheumatic attack appeared on October 1. It was mild but definite. The occurrence of subsequent rheumatic attacks was as follows:

1930				1931							
Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.
	*+				+	+, +, +, ++, ++, ++++	+++, ++++, +, +			+	

\* + mild or questionable, ++ moderate, +++ severe.

From the preceding table it can be observed that during 12 months there were thirteen rheumatic attacks among thirty-five subjects; three severe, two moderate, six mild, and two questionable attacks. Six of these occurred during the 2nd week of March. Previous to that time, only two children had experienced symptoms, and these had not been severe. In addition, two children who were discharged during the 2nd week in March developed rheumatism within a fortnight after arriving home. Eight of the eleven definite attacks were

<sup>7</sup> A severe epidemic of pancarditis among rheumatic subjects was described by Boas and Schwartz (13) at Montefiore Hospital in New York City. They observed that no cases developed among the non-rheumatic population and that the outbreak followed a wave of respiratory infections which spread through the wards and was contracted by all groups of patients.

preceded by mild pharyngitis, accompanied by the presence of hemolytic streptococcus in the throat flora. There was in each instance a symptom-free period of from 1 to 3 weeks between the subsidence of the local infection and the development of definite rheumatic manifestations. In several instances, severe epistaxis preceded by 2 or 3 days the recognition of a rheumatic recrudescence. These findings were similar to those of the years 1928-30, in which two outbreaks of rheumatic recrudescences were observed. Each followed a wave of upper respiratory tract hemolytic streptococcus infection detected by daily throat cultures.

A series of recrudescences was closely observed in one individual. This was the only patient at The Pelham Home with evidence of active rheumatism during the fall of 1930. She was also the only patient who experienced respiratory infection. The recrudescences in this individual appeared to be associated with attacks of sinusitis. During the fall months hemolytic streptococcus was found in this patient and occasionally in two girls with large tonsils. On February 28 during a pharyngitis, this organism (Strain RPH1) reappeared after an absence of 6 weeks. This mild infection with hemolytic streptococcus was preliminary to a fulminating rheumatic attack. During the 10 months since the subsidence of acute symptoms, hemolytic streptococcus has not been observed in her throat flora; she has been free of upper respiratory disease; there have been no rheumatic manifestations.

The association of outbursts of recrudescences in rheumatic colonies has been carefully described by Schlesinger (14) and also by Sheldon (15). Collis (5) made bacteriological studies on the outbreak of recrudescences among the rheumatic children in Dr. Sheldon's wards at the Cheyne Hospital. He found that the organism causing the epidemic was hemolytic streptococcus, and it is shown that only after infection with this organism did relapses occur during these 4 months. A small epidemic of upper respiratory infection termed "colds" and associated with the pneumococcus produced no effects.<sup>8</sup>

These findings of English physicians and the observations at The Pelham Home for girls with rheumatism indicate the close relationship

<sup>8</sup> Through the kindness of Dr. W. R. Collis, two of the throat strains from this epidemic (C1 and C2) have been studied. They are reported in Paper II, page 649.

between outbreaks of recrudescences among isolated groups of rheumatic subjects and the appearance of hemolytic streptococcus in the throat flora of the individuals.

2. *The Relationship of Several Recrudescences in the Rheumatic Family to Outbreaks of Hemolytic Streptococcus Infection.*—That several cases of rheumatism may appear almost simultaneously in a single household has been repeatedly observed. In a previous report (6),<sup>9</sup> such a family epidemic was described and its relationship to an outbreak of hemolytic streptococcus pharyngitis was indicated. Subsequently, three similar family epidemics have been observed clinically and by bacteriological study.

*Family W.*—The father was known to have had rheumatic fever; the mother was thought to be a non-rheumatic subject. The first daughter, C., was admitted to the Presbyterian Hospital at the age of 7. Because of frequent epistaxis, during the 3rd year of life, her tonsils were removed at the age of 4. Mild muscle pains were frequent, and then in 1925, following cervical adenitis, she developed polyarthritis. While under observation in this study she experienced frequent attacks of chorea but escaped severe heart disease. Three more children were born, and their progress observed since infancy. In June, 1930, the first son, Ch., contracted a sore throat; the rheumatic sister was away at a convalescent school, but the second boy was at home. The oldest boy developed pancarditis and was rushed to another hospital, where no throat cultures were obtained. The second brother experienced severe epistaxis at this time and the throat cultures contained hemolytic streptococcus in predominance. During the winter of 1931, C. contracted hemolytic streptococcus pharyngitis while at The Pelham Home and experienced a recrudescence 10 days later. The three oldest children in the city remained quiescent. In the spring of 1931 when the four children were at home together, a respiratory infection was followed by chorea in C., carditis in Ch., erythema marginatum in E. and subcutaneous nodules in A. Following this, it was possible to obtain throat cultures only on C.; hemolytic streptococcus was again recovered. In the fall C. returned to The Pelham Home, where her throat became free of hemolytic streptococcus and her chorea subsided. In Jan., 1932, Ch. again contracted a respiratory infection. The other members escaped, and their throat flora did not contain hemolytic streptococcus. This organism (Strain R29) was, however, present in Ch.'s throat, and its advent was followed by a fulminating, probably fatal, rheumatic attack.

*Family Al.*—Consists of three daughters. Two of them had rheumatism and were observed at The Pelham Home in 1928 and 1929. During a wave of hemolytic streptococcus infections in 1929 both had this organism in the throat flora

<sup>9</sup> Coburn (6), p. 186.

and both experienced mild recrudescences. The youngest girl, S., followed similar hemolytic streptococcus infection, developed evidences of active rheumatism in the spring of 1930 and the winter of 1931. The second daughter seemed to escape infection and no evidence of an active process was detected. On Jan. 9, 1932, hemolytic streptococcus appeared in their home. The oldest sister, M., contracted pharyngitis, and cervical adenitis. She is a non-rheumatic subject. There were no sequellae. On Jan. 10, 1932, A. contracted pharyngitis. Hemolytic streptococcus (Strain R30) which had been absent for 2 years, appeared as the predominating organism in the throat flora. Local symptoms subsided, and the organism disappeared in 5 days. After 2 weeks of good health, this patient was found to have a temperature of  $102^{\circ}$  without symptoms. She was admitted to the hospital where, while resting in bed, she rapidly developed carditis and polyarthritis. On Jan. 23, 1932, S., the youngest sister, contracted a severe "sore throat." Hemolytic streptococcus, which had been absent from the throat flora for 8 months, appeared as the predominating organism (Strain R31); after 1 week's convalescence, she developed carditis and was admitted to the hospital.

*Family G.*—Consisted of a mother and seven children, living under the extreme conditions of destitution. During the spring of 1929, shortly after the period of observation began, the mother, one boy, and one girl suffered, in addition to the family epidemic of laryngitis, manifestations of rheumatic fever. Hemolytic streptococcus was prominent in the throat flora at that time. The daughter, L. G., was taken to The Pelham Home, where daily throat cultures were obtained. For 3 months she harbored hemolytic streptococcus, and mild rheumatic symptoms persisted. During the next 3 months she was symptom-free, and the throat flora contained no hemolytic streptococcus. In contrast, the brother who had been left in the home environment contracted the respiratory diseases epidemic in the house and experienced active rheumatism. Hemolytic streptococcus was frequently recovered. After the daughter's throat flora had

CHART 2. Patient L. G. In this chart and Chart 3, the same set of symbols are employed. The incidence of organisms in the throat flora from right (R) and left (L) sides is represented as follows:

- = predominating.
- = prominent.
- ++++ = plentiful.
- ++ = moderate.
- +
- = rare.

The clinical symptoms and signs are valued as follows:

- ++++ = extreme.
- +++ = marked.
- ++ = moderate.
- +
- = mild.
- = absent.

The simple linear curve indicates the changing condition of a rheumatic girl, a convalescent home and while living in a rheumatic household.





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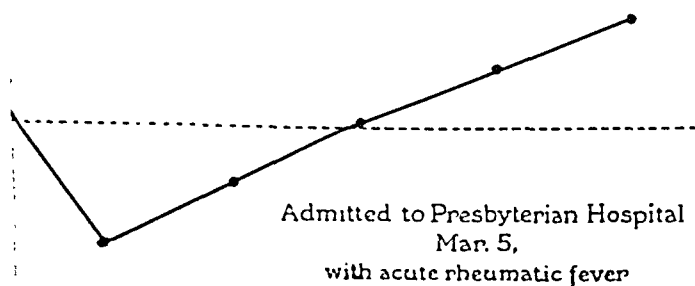
## 1930

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At The Pelham Home										Remarks
1931										
Mar. 4		Apr.		May		June		Oct.		
R	L	R	L	R	L	R	L	R	L	
+	+									During the spring months of 1929, the patient carried hemolytic streptococcus in the throat. This organism then disappeared and was not recovered until Feb., 1931, when for a period of 10 days it was prominent in the pharyngeal flora
○	○	⊙	⊙	⊕	⊕	⊕	⊕	⊕	⊕	
○	○			++	++	++	++	++	++	
+	+	+	+					+	+	
+++	+++	++	++	+	+	+	+	+	+	
Cervical adenitis		•		•		•		•		During the spring and summer of 1929, this patient experienced vague symptoms and only fair health. While at The Pelham Home she improved and after 2 yrs. appeared in good health when in Feb., 1931, she contracted a respiratory infection. This was followed by a severe rheumatic attack
		•		•		•		•		
		•		•		•		•		
104		99		100		100		100		
		87		91		103		114		
+		•		•		•		•		
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1929

Organisms													
	Jan.		Feb.		Mar.		Apr.		May		June		R
	R	L	R	L	R	L	R	L	R	L	R	L	
Staphylococcus aureus.....											++	+	
Staphylococcus.....													
Staphylococcus.....	○	○											
Staphylococcus.....													
Staphylococcus viridans.....	●	●	●	●	●	●	●	●	●	●	●	●	●
Streptococcus.....			+	+	++	++					+	+	
Streptococcus albus.....													
Streptococcus aureus.....				○	+	+	+	+			+		
Streptococcus citreus.....													
Streptococcus.....													
Streptococcus "X".....					+	+							
Negative cocci.....	+++	+++			+	+							
Gram-negative.....													
on "Sore throat".....	●		●		●		●		Laryngitis				
on "Cold".....	●		+		●		●		+				
on "Grippe".....	●		●		●		●						
Temperature, °F.....	98		99		98		99		99		99		
Weight, lbs.....	63		65				69		68		68		
Appetite.....	●		●		●		●		●		●		
Sleep.....	●		●		●		●		●		●		
Nausea.....	●		●		+		●		●		●		
al Vomiting.....	+		+		●		●		●		●		
al Pallor.....	●		●		●		●		●		●		
Fatigue.....	●		●		●		●		●		●		
Epistaxis.....	●		●		●		●		●		●		
Muscle pains.....	+		+		+		+		●		●		
Joint pains.....	●		●		●		●		●		●		
Abdominal pains.....	●		●		●		●		●		●		
Tachycardia.....	100		100		96		110		100		96		
Cardiac pain.....	●		●		●		●		●		+		
Palpitation.....	●		●		●		●		●		●		
Dyspnea.....	●		●		●		●		●		●		
Orthopnea.....	●		●		●		●		●		●		
Headaches.....	●		●		●		++		●		+		
Nervousness.....	+		●		●		●		●		●		
Bed wetting.....	●		●		●		●		●		●		
Twitching.....	●		●		●		●		●		●		
Sweating.....	●		●		●		●		●		●		
Erythema.....	●		●		●		●		●		●		
ical Throat.....	Teeth		Teeth		Teeth		Teeth		Teeth		Teeth		
ical Cervical node.....	+++		+++		++		++		++		++		
ical Heart status.....	+		+		+		+		+		+		

↑  
Two molar teeth

Excellent

Good

Average health

Indisposed

Sick

Very sick

Critically ill

Developing abscess

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R
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○
○
+

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04

been free of hemolytic streptococcus for 13 months, and after she had been entirely symptom-free for 11 months, while living with her family, she contracted pharyngitis which was epidemic in the home. At that time, hemolytic streptococcus (Strain R17) appeared in large numbers in the throat flora. The brother and mother also experienced this pharyngitis and mild rheumatism. 10 days after the arrival of this organism in the daughter's throat, she experienced a fulminating rheumatic attack. For the subsequent 6 months following recovery, she has remained symptom-free and this pathogenic agent has not reappeared. In all, more than 600 throat cultures were made on the daughter during 2 years, at her own home and at The Pelham Home. There was a close relationship between the family epidemics of hemolytic streptococcus pharyngitis and recrudescences of rheumatic disease in these three members. By separating the daughter and son it was possible to demonstrate that a recrudescence occurred only in the individual with a hemolytic streptococcus throat infection. By bringing them together it was striking that, following a household epidemic of hemolytic streptococcus pharyngitis, each member developed a rheumatic attack.

In the first family, the initial appearance of rheumatism was observed in three members. In the second and third families, recrudescences of the disease were observed under a variety of conditions, and careful studies of the throat flora were made. The one constant factor in these outbreaks of recrudescences in rheumatic homes was their relationship to family epidemics of hemolytic streptococcus throat infection.

3. *The Relationship of Recrudescences in the Rheumatic Subject to the Ecology of Hemolytic Streptococcus.*—The findings in a group of rheumatic individuals studied in New York before and after transplantation to Puerto Rico were described in a previous report (6).<sup>10</sup> It was pointed out at that time that recrudescences of rheumatism among the group returning to New York City closely followed the arrival of hemolytic streptococcus in the pharyngeal flora. One of these individuals escaped infection, however, until February, 1931. His story in brief is as follows:

*E. C., No. 300309.*—The patient is a 16 year old boy who had been under observation for 4 years. During two previous recrudescences, hemolytic streptococcus had been recovered from the throat flora. This organism was not found in the pharyngeal cultures from Dec., 1928, the date of sailing for Puerto Rico, until Feb. 14, 1931. During this period of 26 months his rheumatic process appeared entirely quiescent. On Feb. 14 he experienced a "bad cold;" hemolytic streptococcus (Strain R15) reappeared at this time in large numbers. Although he remained in bed, a severe rheumatic recrudescence manifested itself exactly 11

<sup>10</sup> Coburn (6), p. 214.

days after the respiratory tract infection. Since recovery he has remained symptom-free and hemolytic streptococcus has not been found again in his pharynx.

In contrast, two children who experienced severe and frequent bouts of rheumatic fever before going to Puerto Rico have subsequently remained free of respiratory infections. Hemolytic streptococcus has not appeared active in their throats during the past 3 years, and the disease process has remained clinically quiescent. These individuals studied while in the tropics and in New York demonstrated the close relationship of the rheumatic recrudescence to the geographical distribution of hemolytic streptococcus.

4. *The Relationship of Outbreaks of Recrudescences in Ambulatory Rheumatic Subjects to the Incidence of Hemolytic Streptococcus.*—Continuous clinical and bacteriological observations were made on a group of ambulatory rheumatic subjects, 165 of whom had been studied for at least 3 years. As in previous fall seasons, there was little evidence of rheumatic activity in October, 1930. Although experiencing three waves of "common colds" all but three patients were symptom-free until the middle of February, 1931. Within the subsequent 6 weeks, however, ten of the group developed severe recrudescences. These recrudescences in the spring of 1931 were preceded by a wave of hemolytic streptococcus pharyngitis, which also evidenced itself simultaneously among the other groups under observation.

*Monthly Incidence of Acute Rheumatic Attacks among Ambulatory Rheumatic Subjects*

1930				1931							
Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.
* + + + +		+			++ , ++ , ++ , ++	++ , + , + + + ++        ++ , + + + + ,        + + + + + + ,        + + + , + +		++ ,        + + + + + +			

\* + mild attack, ++ moderate attack, +++ severe attack.

CHART 3. Patient E. C. This chart illustrates the course of a rheumatic boy, before, during and after living in the tropics. The throat flora, the clinical picture and a simple linear curve showing the patient's condition are represented with the same symbols as used in Chart 2.





days after the respiratory tract infection. Since recovery he has remained symptom-free and hemolytic streptococcus has not been found again in his pharynx.

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*Monthly Incidence of Acute Rheumatic Attacks among Ambulatory Rheumatic Subjects*

1930				1931							
Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.
*+++		+			++, ++, ++, ++	++, +, +++ ++ +, +++ +++, ++ ++	+++ +, +++ +++	++ +++	+++		

\* + mild attack, ++ moderate attack, +++ severe attack.

CHART 3. Patient E. C. This chart illustrates the course of a rheumatic boy, before, during and after living in the tropics. The throat flora, the clinical picture and a simple linear curve showing the patient's condition are represented with the same symbols as used in Chart 2.





Twenty of the group had well defined infections of the pharynx, and in these, throat cultures plated on fresh rabbit blood agar demonstrated the presence of hemolytic streptococcus. Salicylates were not administered before the onset of the rheumatic attack because of the possibility of masking symptoms and preventing definite interpretation of the clinical changes. The histories of consecutive cases of rheumatism, and the findings in these illustrative individuals are described in brief.

*A. N., No. 71362.*—The patient is a boy of 13, who has been observed for 3 years. He had previously experienced repeated rheumatic attacks, following hemolytic streptococcus pharyngitis. On Jan. 27, 1931, after having been symptom-free for 1 year, he contracted a "sore throat" with hemolytic streptococcus appearing as a prominent organism (Strain R8). This was followed by a severe rheumatic recrudescence, which persisted for 5 months. Hemolytic streptococcus had been absent from the throat cultures previous to the onset of sore throat and has not been recovered subsequently.

*R. G., No. 59498.*—The patient is a man of 22, who has been under observation for several years. He had been symptom-free for 5 years. On Jan. 1, 1930, he contracted a "sore throat." At that time hemolytic streptococcus which had been previously absent from the throat flora, appeared in moderate numbers (Strain R6), persisted for 6 weeks and has not been subsequently recovered. Exactly 3 weeks after the onset of pharyngitis, he experienced a mild rheumatic attack which persisted for about 1 month.

*E. C., No. 81233.*—The patient is a girl of 9, who has been under observation for a period of 3 years. In Jan., 1931, she contracted a "sore throat" which was followed by a mild rheumatic attack. Hemolytic streptococcus, which previously had not been recovered from the patient's pharynx, appeared as the predominant organism (Strain R10) at the day of onset of infection, persisted for 1 week and then disappeared. This organism was associated with infection in other members of the family and has not been subsequently recovered from the patient's throat flora.

*P. Mc., No. 71128.*—The patient is a boy of 18, whose three sisters living in different parts of New York have been observed during rheumatic attacks. On Nov. 11, 1930, he contracted a "sore throat" at which time hemolytic streptococcus (Strain R3) was present in large numbers. 3 weeks later he developed poly-arthritis and a presystolic murmur.

*W. P., No. 257703.*—The patient is a 19 year old clerk, under observation for 2 years. He appeared in perfect health, when on Feb. 26, 1931, he contracted a "cold." At that time his pharynx was markedly injected and the throat culture contained hemolytic streptococcus in almost pure culture (Strain R18). This organism, which had previously been absent, persisted for 12 days. 10 days after the local infection, he developed a severe rheumatic attack which necessitated treatment in the hospital for 13 weeks.

*J. D., No. 61215.*—The patient is a 12 year old boy, under observation for 7 years. On Apr. 15, 1931, he contracted a "sore throat." At that time, hemolytic streptococcus, which had not been recovered from the pharynx in 2 years, appeared in almost pure culture (Strain R23), persisted for 1 week and then disappeared. Shortly after the local infection subsided, he developed a severe rheumatic attack which necessitated bed care for several months.

*G. R., No. 73773.*—The patient is a 21 year old college student, under observation for 3 years. On Feb. 2, 1931, she contracted a "sore throat." At that time hemolytic streptococcus, which had not been recovered from the pharynx in 18 months, appeared in almost pure culture (Strain R11), persisted for 3 days and then disappeared. The local infection was followed by a mild rheumatic attack.

*M. R., No. 83325.*—The patient is a housewife of 33, who has been under observation for 2 years. On Feb. 5, 1931, she contracted a "sore throat." At this time, hemolytic streptococcus appeared as the predominating organism (Strain R12), persisted for 2 weeks and then disappeared. 10 days after the local infection she developed a moderately severe rheumatic attack.

*J. Z., No. 290435.*—The patient is a 32 year old cook who has been under observation for a few months. On Mar. 11, 1931, he contracted a "sore throat." At this time hemolytic streptococcus was the predominating organism (Strain R20). His tonsils were later removed and this organism recovered in pure culture. The local infection was followed by a mild rheumatic attack, which necessitated hospital treatment.

*M. O., No. 82235.*—The patient is an 18 year old maid who has been under observation for 1 year. She was in good health when on Feb. 21, 1931, she contracted a "sore throat." At that time hemolytic streptococcus, which had previously been absent, appeared in large numbers in the pharyngeal cultures (Strain R35). The organism persisted for a few days and then disappeared entirely. 3 weeks after the onset of the local infection she developed a moderately severe rheumatic attack. This association of recrudescences with hemolytic streptococcus infection of the throat was observed in this individual in the winters of 1929, 1931 and 1932.

*F. B., No. 70078.*—The patient is a schoolboy of 11, who has been under observation for 5 years. Throughout the entire years of 1930 and 1931 he experienced rheumatic activity and hemolytic streptococcus was always present in the pharyngeal cultures. Tonsillectomy was performed on May 1, 1931, and hemolytic streptococcus recovered in pure culture from the tonsils (Strain R22). 10 days later he developed a fulminating attack of rheumatism. This organism has not been recovered subsequently and his disease process has remained quiet.

*V. D., No. 59760.*—The patient is a girl of 16 who has been under observation for 5 years. She had been observed throughout six mild recrudescences. On Mar. 1, 1931, she contracted a "sore throat." At this time hemolytic streptococcus, which had not been recovered for 11 months, appeared in almost pure culture (Strain R21). This was followed by a moderately severe rheumatic attack 9 days after the local infection. She subsequently remained symptom-free and hemolytic streptococcus has not yet again been recovered.

*C. M., No. 285560.*—The patient is a 12 year old girl observed during the period of 1 year. Following discharge from The Pelham Home, she contracted a "sore throat" and 2 weeks later, Nov. 21, 1931, she was admitted to the hospital with acute rheumatism. After 3 months' rest in bed, the tonsils were removed. 48 hours later, she developed a severe recrudescence. Each tonsil contained hemolytic streptococcus in pure culture (Strain R26).

*R. B., No. 71809.*—The patient is a 15 year old boy who has been under observation for 4 years. During 1928 and 1929 he carried hemolytic streptococcus in his throat and experienced mild rheumatic symptoms. In Oct., 1931, he appeared in better health than ever in the past. He had been entirely symptom-free for more than 1 year, and hemolytic streptococcus had not been recovered from his throat in 18 months. On Nov. 30 he contracted a "bad cold." Hemolytic streptococcus (Strain R25) reappeared in almost pure culture. A rheumatic recrudescence followed 1 week later. After 3 months of rest the tonsils were removed, and the identical type of hemolytic streptococcus was present in predominance. 4 hours later he experienced an exsanguinating epistaxis. This was followed 2 days later by a severe recrudescence of the rheumatic process.

*I. P., No. 241826.*—The patient is a young West Indian negro housewife, previously admitted with rheumatic heart disease. While under observation for 2 years, hemolytic streptococcus was not recovered from her throat flora. On Nov. 16 she contracted a "sore throat" with hemolytic streptococcus in predominance. 10 days later she was admitted to the hospital with pancarditis. The tonsils were removed on Jan. 20. They contained hemolytic streptococcus in almost pure culture. This organism was identical with the throat strain of Nov. 16 (Strain R27). 6 days later she developed a recrudescence.

*T. F., No. 307511.*—The patient is a girl of 6 who after 8 months rest in bed appeared to have recovered from a severe rheumatic attack. She was ready for convalescent care at The Pelham Home when on Feb. 10, the house officer in charge of her ward developed hemolytic streptococcus pharyngitis. The following day, she contracted a "sore throat." Pharyngeal cultures on Feb. 12 contained hemolytic streptococcus in predominance. This organism (Strain R34) disappeared after a few days, and the patient recovered rapidly. On Feb. 20, after 6 days of normal temperature, the patient developed a fulminating rheumatic attack with pancarditis.

*F. R., No. 68034.*—The patient is a 15 year old school boy under observation for 4 years. He had experienced typical attacks of rheumatic fever in 1924 and 1926, for which his tonsils had been removed. Although stigmatized with mitral stenosis he remained symptom-free until May, 1928, when he developed a recrudescence of his disease following hemolytic streptococcus pharyngitis. For 3 years he enjoyed excellent health, except for gonococcus urethritis. On Mar. 10 he contracted pharyngitis, and hemolytic streptococcus (Strain R36) reappeared in his throat for the first time in 3 years. On Mar. 19, 7 days after the subsidence of the local infection, he again became ill with typical acute rheumatic fever.

These histories illustrate in brief the close association between hemolytic streptococcus and acute attacks of rheumatic fever. During March and April the incidence of recrudescences among ambulatory subjects in good health was high. These attacks followed closely the hemolytic streptococcus throat infections which appeared in epidemic proportions in New York during the weeks previous.

In the preceding sections, a series of observations are detailed to illustrate the influence of hemolytic streptococcus on the epidemiology of rheumatic fever. Outbreaks of this disease are striking but not common; however, the frequency of recrudescences in the rheumatic subject is one of the striking characteristics of the morbid process. Because of their importance, these sporadic relapses of rheumatism have been closely observed, both by clinical methods and by studying the throat flora of these individuals during health and disease. It has been noted that in the City of New York, hemolytic streptococcus is not a part of the normal throat flora. Its appearance in the pharynx in large numbers has usually been associated with local infection, which in the rheumatic subject has frequently but not always been followed by a recrudescence. The previous (6)<sup>11</sup> and recent findings in a group of ambulatory rheumatic subjects are summarized in Table I.

TABLE I  
*The Relationship of Infection with Hemolytic Streptococcus to Recrudescences of Rheumatism*

	Cases for 4 yrs.
1. Appearance of hemolytic streptococcus in pharyngeal cultures shortly before the onset of the rheumatic attack.....	77
2. Absence of hemolytic streptococcus from the pharyngeal cultures and absence of rheumatic manifestations.....	71
3. Return of rheumatic manifestations without hemolytic streptococcus being obtained from the pharynx.....	17
4. Patients with undetermined relationship.....	32
5. Appearance of hemolytic streptococcus in predominance on pharyngeal culture without development of rheumatic manifestations....	25

Two things appeared definite in the study of these patients: first,

<sup>11</sup> Coburn (6), p. 168.

in the rheumatic subjects who remained free of upper respiratory tract infection, the morbid process seemed in almost all cases clinically quiescent; second, in those individuals who contracted a pharyngitis before the recrudescence, the throat flora included hemolytic streptococcus during the period of infection. As in scarlet fever (16) this organism usually disappeared from the throat flora in 2 weeks. Even in most of the patients with extremely severe rheumatism, the pharyngeal infection was mild and transient. In many instances, this pathogenic agent, although present in nearly pure culture at the onset of pharyngitis, was inconspicuous during the quiescent period and absent when the rheumatic process manifested itself.

A less well defined relationship appeared in three other groups. In seventeen patients, the infection preceding the rheumatic attack did not occur in the pharynx, and the throat flora showed nothing abnormal. Thirty-two patients had vague complaints and frequently carried hemolytic streptococcus in the throat flora. In these, it was not possible to date the onset of infection nor determine satisfactorily the reactivation of the disease process. Twenty-five patients seemed to escape a recrudescence following hemolytic streptococcus infection. Three of these, under close observation, were found to experience pyrexia ( $102^{\circ}$ ) during the 2nd week after infection. Whether rheumatic attacks were aborted or whether the rheumatic process became active, but too insidious for clinical recognition, is not determined.

Finally, six consecutive hemolytic streptococcus infections, occurring in a period of 2 weeks among the ambulatory group, illustrate the relationship of this organism to the rheumatic attack. The clinical records of these patients are in brief as follows:

*H. R., No. 82718.*—This 14 year old boy with rheumatic heart disease had remained almost symptom-free for 3 years. Although he contracted "colds," pathogenic agents were not recovered from the pharyngeal flora until Apr. 14. On that day he experienced a "sore throat" with fever  $102^{\circ}$ , and hemolytic streptococcus appeared as the predominating organism. All symptoms, the infectious agent and the leucocytosis disappeared in 3 days. During the next 7 days the patient, although closely observed, seemed in excellent health. On Apr. 28, a change took place. He experienced malaise; there was striking alteration in the electrocardiogram, characterized by the sudden development of prolonged conduction time with P-R interval equal to 0.23 second and an elevation of the S-T interval. At the same time, although the pulse and temperature remained normal,



the Addis red cell count rose precipitously from normal to 20 millions in 12 hour urine excretion and leucocytosis of 12,000 developed.

14 days after hemolytic streptococcus (Strain R45) infection, the findings indicated reactivity of the rheumatic process; however, the patient was entirely unaware of these changes which were detected only by laboratory studies. 1 month later he was readmitted to the hospital because of myocardial insufficiency.

*M. H., No. 292652.*—This 14 year old girl had been symptom-free for 12 months. On Apr. 29 she contracted a "sore throat" with fever of 102°. Throat cultures, two each week for 12 months, had up until this day revealed no pathogenic organisms; however, with the onset of pharyngitis, hemolytic streptococcus appeared prominent in the flora. 3 days later, the temperature and white blood count were normal. On the 10th day after subsidence of the local infection, with normal pulse and temperature, she developed mild joint pains, especially about the left shoulder; the white blood count rose to 14,000; no other rheumatic manifestations developed.

This individual experienced a rather severe throat infection with hemolytic streptococcus (Strain RPH2) on April 29. On May 12 a change took place. The patient was under close observation and a steady rise in the leucocyte count and mild polyarthritis were detected. This attack was so slight that, although recognized by the physician, it would have probably been overlooked by the patient.

*L. K., No. 277788.*—This 13 year old girl with mitral stenosis had experienced no rheumatic manifestations for 9 months when on May 1 she contracted a "sore throat" with fever of 103°. No pathogenic organisms had been found in the throat flora, although two cultures were obtained each month, until the 1st day of symptoms, when hemolytic streptococcus appeared in almost pure culture. After 4 days, all symptoms and this organism disappeared; however, a slight elevation of temperature and leucocyte count persisted. On May 11, the temperature rose to 101°; there were mild precordial twinges; the leucocyte count rose from 9,400 to 12,000 with an increase in polymorphonuclears from 68 to 81 per cent. This was followed by headache, mild joint pains and further elevation of body temperature.

On the 12th day after the onset of hemolytic streptococcus (Strain R48) throat infection, this patient experienced a series of changes which were characterized by an abnormal temperature, leucocyte count and by mild aches in the region of the precordium and joints. They were recognized, probably only because the patient was under close observation in the hospital.

*M. K., No. 280024.*—This 14 year old girl with rheumatic heart disease had been symptom-free for 9 months. Throat cultures had been obtained twice a week or twice a month during this period, and hemolytic streptococcus was not recovered until the onset of a "cold" and mild "sore throat" on May 2. The local infection was transient; she was kept in bed only 1 day. On May 10 she developed a typical rheumatic attack with fever  $102^{\circ}$ , leucocytosis and migrating polyarthritis with the usual response to sodium salicylate.

This individual, following a mild pharyngitis with hemolytic streptococcus (Strain R49), developed a moderately severe, easily recognized attack of rheumatism.

*E. L., No. 298005.*—This 16 year old boy with rheumatic heart disease appeared symptom-free until Apr. 29 when he contracted a "bad cold." Previous throat cultures obtained each month had not contained hemolytic streptococcus. However, with the onset of the respiratory infection this organism predominated the pharyngeal flora. On May 5, 3 days after recovery from the local infection, there was a rise in body temperature to  $101^{\circ}$  with a tachycardia of 140. While at rest in bed, the patient developed a severe rheumatic attack with epistaxis, polyarthritis and carditis.

This boy, recovering from hemolytic streptococcus (Strain R46) pharyngitis, developed fever, epistaxis and tachycardia, which were followed by the well recognized picture of rheumatic fever with myocardial insufficiency.

*S. S., No. 339869.*—This 19 year old girl with mitral stenosis was almost entirely symptom-free for 4 years, when on Apr. 22 she contracted "grippe" and a "bad cold." This was accompanied by the advent of hemolytic streptococcus, not recovered in over 3 years from her pharyngeal flora. The local infection was severe and was followed by cervical adenitis. On May 4, 1 week after recovery, she seemed in excellent condition; however, on May 11 she became nervous. At this time her temperature was  $101^{\circ}$ . She was not aware of having rheumatism. During the following 48 hours, the temperature, pulse and leucocyte count rose steadily. She developed a fulminating rheumatic attack.

This individual with a 4 year symptom-free period experienced an intense rheumatic attack 10 days after recovery from infection of the upper respiratory tract with *Streptococcus hemolyticus* (Strain R47).

These patients illustrate the effect of six consecutive hemolytic streptococcus infections among a group of ambulatory rheumatic subjects. Each individual was in the adolescent period; each lived a

few miles from the other; each had escaped respiratory disease for months; in each the throat flora had been free of hemolytic streptococcus for 1 year or longer; each contracted a pharyngeal infection during the last 10 days of April; in each the advent of hemolytic streptococcus was recognized at the onset of the local infection. In the first patient, the laboratory determinations showed evidence which suggested a recrudescence of the rheumatic process. In the second and third, with close clinical observation, it was possible to detect signs which the patient may have overlooked. In the last three individuals, typical well recognized rheumatic attacks followed about 48 hours after the rise in temperature and pulse rate were observed by the physician. The findings in this small group are representative of the observations made over a period of 4 years; namely, that following respiratory infection with *Streptococcus hemolyticus* the rheumatic subject usually experiences a clinical change. This change, in most instances, is characterized by symptoms which indicate activity of the disease process, but in some individuals is detected only by the objective findings of the physician.

#### SUMMARY

Certain factors of climate are favorable to streptococcus respiratory diseases. In those tropical environments where hemolytic streptococcus is unusual in the throat flora, scarlet fever is unknown and rheumatic fever rare. In New York City, however, following epidemic waves of pharyngitis with hemolytic streptococcus the incidence of rheumatic fever rises precipitously. The correlation between the geographical distribution of hemolytic streptococcus and rheumatic fever is a definite one.

Furthermore, in New York City during the seasons of the year in which hemolytic streptococcus is seldom recovered from the pharynx, acute attacks of rheumatism are unusual. Corresponding to the seasonal rise in hemolytic streptococcus infections, the curve of incidence of acute rheumatism shows a similar form.

Among the children of wealthy patients, enjoying great protection, hemolytic streptococcus has been recovered infrequently from the throat, and rheumatism has not been encountered during this study. Among the poor under observation in New York City, however, the

organism is found frequently in the pharyngeal flora, and rheumatic fever is common. The findings suggest that poverty and unhygienic living conditions favor both the activity of hemolytic streptococcus in the throat and the incidence of rheumatic fever.

Moreover, localized outbreaks of rheumatism have been observed frequently following epidemics of "sore throat." Bacteriological studies of these upper respiratory infections demonstrate a close relationship between the advent of hemolytic streptococcus in the throat flora and the outbreak of rheumatic fever in susceptible individuals.

In addition to these studies of streptococcus infections and their relationship to the development of rheumatic fever, observations of the rheumatic patient add further emphasis to this association. First, among a group of rheumatic children in an isolated environment, reactivation of the rheumatic process has been recognized only following the advent of hemolytic streptococcus in the throat flora.

Also, an investigation of families in which several members have rheumatic heart disease has led to the same conclusion. Recrudescences of the disease have been observed under a variety of conditions among these individuals. However, the one constant factor in the outbreaks of recrudescences in rheumatic homes is their association with family epidemics of hemolytic streptococcus infection.

Moreover, by studying rheumatic patients before, during and after transplantation to a tropical environment, it has been possible to demonstrate a close relationship between activity of the disease process and infection with hemolytic streptococcus. While the rheumatic patients remained in the tropics this organism was not recovered from the pharyngeal flora, and the disease process seemed quiescent. On return to New York City, those individuals who have escaped respiratory infection have remained symptom-free. However, of those who have contracted hemolytic streptococcus pharyngitis, each has developed a rheumatic attack within 3 weeks after infection.

Finally, extensive bacteriological studies made in ambulatory rheumatic subjects over a period of 4 years have demonstrated that the individuals who escape respiratory disease remain free of rheumatic manifestations. On the other hand, the majority of rheumatic patients who contract hemolytic streptococcus pharyngitis experience shortly afterward a definite recrudescence of their disease. In con-

clusion, there is a close relationship between respiratory infection with hemolytic streptococcus and activity of the rheumatic process in susceptible individuals.

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# STUDIES ON THE RELATIONSHIP OF STREPTOCOCCUS HEMOLYTICUS TO THE RHEUMATIC PROCESS

## II. OBSERVATIONS ON THE BIOLOGICAL CHARACTER OF STREPTOCOCCUS HEMOLYTICUS ASSOCIATED WITH RHEUMATIC DISEASE

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In order to investigate further the nature of the hemolytic streptococcus associated with acute rheumatism (1), a study has been made to determine the biological character of the organisms encountered. The observations include: a study of toxin production; neutralization of toxin by a standard antitoxin; the question of the existence of specific types of these streptococci, and their relationship to scarlatinal strains of hemolytic streptococcus.

*1. General Characteristics.*—In Table I the general characteristics of the organisms studied are presented. The cultural characteristics represent the appearance of surface colonies at 72 hours seen through a Zeiss plate microscope. In determining the fermentation reactions Holman's technique was employed. As indicators brom-cresol purple 0.002 per cent and methyl red 0.04 per cent were used.

The general characteristics are detailed in Table I. With few exceptions,<sup>1</sup> no unusual phenomena of growth were encountered. Ac-

<sup>1</sup> Phosphate broth inoculated with the organisms in Table I in 24 hours showed flocculent or granular growth in suspension and formed varying amounts of sediment; whereas 0.1 per cent dextrose broth in 24 hours deposited an abundant sediment but left a clear supernatant. No. R4 was the only exception. It was isolated from the interior of a tonsil and grew with great difficulty at first. After several passages through blood broth and on blood agar plates, it multiplied but without visible growth in phosphate broth. Not until the tenth passage in the phosphate broth did growth appear; this was a very fine dusty sediment. At no time did it afford a yield sufficient for making HCl extract.

The organisms on artificial medium with three exceptions remained constant

cording to Holman's classification, more than 70 per cent of the organisms associated with rheumatic fever have been *Streptococcus pyogenes*. Others in order of frequency have been *Streptococcus infrequens*, *Streptococcus equi* and *Streptococcus hemolyticus* III.

2. *Toxin Production*.—In order to obtain information concerning the capacity of these organisms to produce toxin, three groups of strains were sent to the New York State Department of Health. There, through the kindness of Miss Mary Wheeler and Miss Mary Kirkbride, the toxin production of the strains was determined by testing the filtrates from 48 hour broth cultures intracutaneously in goats. The strains included:

Group A, old laboratory throat strains from individuals who had scarlatina followed by severe rheumatic disease (R1 and R2).

Group B, organisms obtained from the throats of patients admitted to the hospital with rheumatic disease (Strains R7, R9 and R13).

Group C, organisms from the pharynx of rheumatic individuals obtained at the time of sore throat. In each instance the infection was followed by definite rheumatic attack. Strains R8, R12, R17 and R18 were from patients who had severe rheumatic manifestations, and Strains R3, R6 and R11 from patients with mild attacks. Strain S9 was associated with moderately severe scarlet fever, which was followed by definite rheumatism with carditis.

The findings are summarized in Table II, *a*.

The toxicity of the strains was determined by testing the filtrates from 48 hour broth cultures intracutaneously in goats. Neutralization tests were also made with an antistreptococcus goat serum produced with Dochez NY5 strain of hemolytic streptococcus. Each filtrate with the exception of the filtrate of RS9 was tested at different times on from two to four goats.

Two filtrates prepared at different times from each of Strains R1, R2, R9 and R13 induced no reaction or only a questionable reaction

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in their appearance. No. R6 developed a variant, a hemolytic streptococcus. The original was a raised olive-brown colony; the variant was flat, without definite center. The two showed a difference in the fermentation of salicin. No. R16 on two occasions developed a variant, which was a methemoglobin former. No. R5 developed during the first 6 months on blood broth a non-hemolytic variant with sugar reactions identical to the original strain.

TABLE I

*Characteristics of Hemolytic Streptococcus from Rheumatic Fever and Scarlatina*

Patient	Strain	Source	Cultural characteristics	Fermentation reactions		
				Lactose	Manitol	Salicin
C.....	*R1	Coburn (1), page 253	Granular	††	—	†
P.....	R2	" (1), " 260	"	†	—	†
P. Mc.....	R3	Paper I, page 623	"	†	—	†
G.....	R6	" I, " 623	"	†	—	†
N.....	R8	" I, " 623	"	†	†	†
C.....	R10	" I, " 623	Finely granular	†	—	†
Ra.....	R11	" I, " 624	" "	—	—	†
Ri.....	R12	" I, " 624	Granular	†	—	†
Cl.....	R15	" I, " 621	Coarsely granular	—	—	†
G.....	R17	" I, " 620	Granular	†	—	†
P.....	R18	" I, " 623	"	†	—	†
Z.....	R20	" I, " 624	Very coarsely granular	—	—	†
D.....	R21	" I, " 624	Finely granular	†	—	†
B.....	R22	" I, " 624	Granular	†	—	†
Do.....	R23	" I, " 624	"	†	—	†
D.....	R4	Acute rheumatism		—	—	†
K.....	R5	" "	Granular	†	—	†
Sa.....	R7	" "	"	—	—	—
St.....	R9	" "	"	†	†	†
C.....	R13	" "	"	†	—	†
H.....	R14	" "	"	†	—	†
M.....	R19	" "	Very finely granular	—	†	†
K.....	R24	" "	Finely granular	†	—	†
C.....	RN1	Paper I, page 615	" "	†	—	†
S.....	RN2	" I, " 615	Granular	†	—	†
St.....	RN3	" I, " 615	Finely granular	†	†	†
M.....	RN4	" I, " 615	Granular	†	—	†
G.....	RPH1	" I, " 618	"	†	—	†
W.....	RG1	" I, " 616	"	†	†	†
B.....	RG2	" I, " 616	"	†	†	†
H.....	RG3	" I, " 616	Coarsely granular	—	—	†
Wa.....	RG4	" I, " 616	" "	—	—	†
Mc.....	RC1	" I, " 618	Granular	—	†	†
C.....	RC2	" I, " 618	"	—	†	†
	S1 (NY5)	Typical scarlet fever	Finely granular	†	—	†
273.....	S2	" " "	" "	†	—	†
B.....	S3	" " "	Granular	†	†	†
N.....	S4	Throat " "	"	†	—	†
	S5	Peritoneum scarlet fever	"	†	—	†
R.....	S6	Throat scarlet fever	"	†	—	†
S.....	S7	" " "	Very coarsely granular	†	—	†
C203.....	S8	" " "	Finely granular	—	—	†
Mc.....	RS9	" " "	Granular	†	—	—
		followed by rheumatism				
	M1 (Rock. S24)		Finely granular	†	—	†

\* The letter R indicates association with rheumatic fever. † — indicates no color change; † indicates moderate or marked change in color.



in the largest dose employed, 0.01 cc. (0.1 cc. of a 1:100 dilution of filtrate).

Filtrates of Strains R3 and R6 were slightly toxic, inducing slight but definite skin reactions in doses of 0.01 cc. and 0.0002 cc., depending on the susceptibility of the animal tested. Both toxins were neutralized by the Dochez NY5 antistreptococcus goat serum.

TABLE II, a

*Toxin Production by Strains of Hemolytic Streptococcus Associated with Rheumatic Fever*

Organism	No toxin production	Moderate toxin production	Strong toxin production	Toxin neutralized by NY5 streptococcus antiserum
R1.....	—			
R2.....	—			
R9.....	—			
R13.....	—			
R3.....		+		+
R6.....		+		+
R7.....			+	+
R8.....			+	+
R11.....			+	—
R12.....			+	—
R15.....			+	+
R17.....			+	—
R18.....			+	—
RS9.....			+	+

Strains R1 and R2 had been on artificial media for a long period of time. Strains R7, R9 and R13 were recovered during attacks of rheumatism. The remainder were cultured from throats during the transient infection preceding the rheumatic attack.

Strain S9 produced a moderately potent toxin neutralized by the NY5 antistreptococcus serum. 0.001 cc. of filtrate induced a strong and 0.0002 cc. a moderate skin reaction.

Strains R7, R8 and R15 produced toxins of high potency. The filtrates of these strains induced definite skin reactions in doses of from 0.00001 cc. to 0.00004 cc., depending on the susceptibility of the animal tested. These toxins were all neutralized by the NY5 antistreptococcus serum.

TABLE II, b

*The Neutralization of Toxins Produced by Strains of Hemolytic Streptococcus Occurring in the Respiratory Infection Preceding Rheumatic Fever*

Skin reactions of strains isolated from the pharynx in			Rheumatic fever										Scarlet fever
Filtrate dilution	Neutralized with equal parts of horse antiserum	Titer of antiserum in units	R28	R36	R37	R38	R39	R43	R47	R48	R49	R50	NY5*
1:100	New York City Scarlatinal Antitoxin	1	++	+	+	+	+	+	+	+	+	+	+
1:100	" " "	5	-	±	+	+	-	+	+	+	+	+	±
1:100	" " State Antistreptococcus Serum	1	-	±	+	+	-	+	+	+	±	+	-
1:100	" " " "	5	-	-	-	±	-	+	±	±	-	-	-
1:500	New York City Scarlatinal Antitoxin	1	±	+	+	+	+	+	+	+	+	+	+
1:500	" " "	5	±	±	±	±	-	+	±	±	±	±	±
1:500	" " State Antistreptococcus Serum	1	-	-	-	±	-	±	±	±	±	±	-
1:500	" " " "	5	-	-	-	±	-	±	±	±	±	±	-
Controls													
Filtrate heated, 1:100 dilution			-	-	-	-	-	-	-	-	±	-	±
Broth, 1:100 "			-	-	-	-	-	-	-	-	-	-	-
New York City Scarlatinal Antitoxin		5	-	-	-	-	-	-	-	-	-	-	-
" " State Antistreptococcus Serum		5	-	-	-	-	-	-	-	-	-	-	-

± indicates erythema; + is a macular lesion over 1 cm. in diameter; ++ represents a papular lesion with erythema more than 1.5 cm. in diameter. All readings were made at 24 hours. The silver fox rabbits employed in this study gave ± reaction and + reaction to 10 and to 100 skin test doses of Dick toxin.

\*This scarlet fever organism was a fresh culture of the standard strain used by the New York State Department of Health.

Strains R11, R12, R17 and R18 produced toxins of equally high potency. A dose of the NY5 antistreptococcus goat serum which completely neutralized the toxins of Strains R7, R8 and R15, had, however, little or no neutralizing activity for the toxins of Strains R11, R12 and R18.

A further investigation was made 1 year later to determine whether these findings of Miss Wheeler were peculiar to a single outbreak of rheumatism. The organisms examined in this second study were obtained in almost pure culture during the pharyngeal infections preceding severe attacks of acute rheumatism. The toxicity of the strains was determined shortly after isolation by testing the filtrates from 48 hour broth cultures on the skin of large silver fox rabbits. Neutralization of the toxins with New York State Antistreptococcus Serum and with the globulin fraction of New York City Scarlatinal Antitoxin was studied. The results are presented in Table II, *b*.

From Table II, *b*, it is seen that strains of hemolytic streptococcus associated with acute rheumatism produce strong toxins; that these toxins in most cases are completely neutralized and in others instances partially neutralized by NY5 antistreptococcus serum; and that these toxins are in most instances not neutralized by the globulin fraction of scarlatinal antitoxin. These findings show that the observations on the toxins produced by strains of hemolytic streptococcus associated with rheumatic fever in New York City are not limited to a single seasonal outbreak.

In summary, these studies indicate that the hemolytic streptococcus, isolated from the throat during the infection which precedes the rheumatic attack, is in most instances a potent toxin producer, and that the majority of these toxins are neutralized by a standard monovalent antiserum.

3. *A Serological Classification of Hemolytic Streptococcus Associated with Rheumatic Fever.*—The most accurate method now available for the classification of streptococcus is one based on the studies of Lancefield (2) who has developed a method for classification of hemolytic streptococcus founded on the precipitin test, using serum from which group antibodies are absorbed. The results obtained agree with those previously determined by agglutination and protection tests. The following technique, which is essentially that of Lance-

field, has been employed in the classification of hemolytic streptococcus associated with acute rheumatism.

*A. Preparation of HCl Extracts.*—HCl extracts were prepared as follows: A strain of streptococcus was cultivated in 20 liters of buffered sodium phosphate broth for 24 hours. The organisms from this material were collected in a Sharpless centrifuge. The material was suspended in 90 cc. of normal salt solution. To this was added 5 cc. of  $N/1$  HCl, making a final concentration approximately  $N/20$  HCl. The suspension in a Pyrex centrifuge tube was immersed in boiling water for 30 minutes and stirred frequently. After cooling, this material was centrifuged. The supernatant fluid was removed and neutralized with  $N/1$  NaOH (indicator phenol red). After standing overnight in the ice box a white fluffy precipitate usually appeared. This was centrifuged down, and the clear supernatant fluid was used as antigen.

*B. Technique of Immunization.*—The rabbit antisera were prepared as follows: Adult brown rabbits weighing between 2 and  $2\frac{1}{2}$  kilos were first tested against protein and polysaccharide fractions of hemolytic streptococcus to rule out the presence of antibodies to streptococcus. Antibacterial sera were then prepared by injecting increasing doses of heat-killed 18 hour broth cultures followed by 18 hour living cultures.

Injectations were given on 4 successive days, followed by a rest period of 3 days.

Generally, the rabbits' precipitin titers were satisfactory after the seventh series of inoculations,<sup>2</sup> but occasionally a long rest period followed by an eighth inoculation was necessary. The immune sera obtained were kept at ice box temperature without the addition of a preservative.

*C. Absorption of Sera.*—Absorption of sera was obtained as follows: Bacteria from 1.5 liters of plain broth culture of heterologous (S24) and a similar quantity of homologous organisms were centrifuged. The organisms in each instance were suspended in 1 cc. of saline and killed by heating at  $60^\circ$  for 1 hour. To each tube of packed bacteria, 3 cc. of immune serum and sufficient saline to give the desired dilution were added. Parallel absorptions were performed at  $37^\circ$  for 10 minutes, with both homologous and heterologous organisms. After centrifugation the supernatant diluted serum was removed, and preliminary precipitin tests set up with

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<sup>2</sup> Series 1-1.0 cc. heat-killed broth culture intravenously.

" 2-2.0 " " " " "

" 3-4.0 " " " " "

" 4-0.5 " living broth culture intraperitoneally.

" 5-1.0 " " " " "

" 6-2.0 " " " " "

" 7-1.0 " " " " "

Trial bleeding 3 days after last injection. Rest period of 6 to 8 wks., if necessary.

Series 8-5.0 cc. living broth culture.

TABLE III  
Precipitin Reactions of Hemolytic *Streptococcus R11*

RHEUMATIC FEVER. II										
HCl extract		Rabbit Serum R11 (diluted 1:3)			Rabbit sera	HCl Extract R11				
		Unabsorbed	Absorbed with strain			Unabsorbed	Absorbed with strain		Heterologous	
			Homol- ogous	Heterologous			Homologous			
R1.....	-	-	-	-	1	#	-	-	-	
R2.....	-	-	-	-	2	+	-	-	-	
R3.....	-	-	-	-	3	+	-	-	-	
R5.....	-	-	-	-	5	+	-	-	-	
R6.....	-	-	-	-	6	-	-	-	-	
R7.....	-	-	-	-	7	-	-	-	-	
R8.....	-	-	-	-	8	-	-	-	-	
R9.....	-	-	-	-	9	-	-	-	-	
R10.....	-	-	-	-	10	+	-	-	-	
R11.....	+	+	+	+	11	+	-	-	-	
R12.....	+	+	+	+	12	+	-	-	-	
R13.....	+	+	+	+	13	+	-	-	-	
R14.....	+	+	+	+	14	+	-	-	-	
R15.....	+	+	+	+	15	+	-	-	-	
R16.....	+	+	+	+	16	+	-	-	-	
R17.....	+	+	+	+	17	+	-	-	-	
R18.....	+	+	+	+	18	+	-	-	-	
R19.....	+	+	+	+	19	+	-	-	-	
R20.....	+	+	+	+	20	+	-	-	-	
R21.....	+	+	+	+	21	+	-	-	-	
R22.....	+	+	+	+	22	+	-	-	-	
R23.....	+	+	+	+	23	+	-	-	-	
R24.....	+	+	+	+	24	+	-	-	-	
R25.....	+	+	+	+	25	+	-	-	-	
RN1.....	+	+	+	+	*1	+	+	+	+	
RN2.....	+	+	+	+	2	+	+	+	+	
RN3.....	+	+	+	+	3	+	+	+	+	
RN4.....	+	+	+	+	4	+	+	+	+	





[illegible]







TABLE III (Concluded)  
Reactions of Hemolytic Streptococci G3 and G4

HCl extract	Streptococcus G3				Streptococcus G4			
	Rabbit Serum G (undiluted)				Rabbit Serum G4 (diluted 1:3)			
	Unabsorbed	Absorbed with strain		HCl extract	Unabsorbed	Absorbed with strain		Heterologous
		Homologous	Heterologous			Homologous	Heterologous	
R1.....	-	-	-	1	-	-	-	-
R2.....	-	-	-	2	-	-	-	-
R3.....	-	-	-	3	-	-	-	-
R5.....	-	-	-	5	±	-	-	-
R6.....	±	-	-	6	-	-	-	-
R7.....	-	-	-	7	+	-	-	-
R8.....	-	-	-	8	-	+	-	-
R9.....	-	-	-	9	±	-	-	+
R10.....	-	-	-	10	±	-	-	-
R11.....	+	+	+	11	+	-	-	-
R12.....	-	-	-	12	+	-	-	-
R13.....	-	-	-	13	-	-	-	-
R14.....	-	-	-	14	+	-	-	+
R15.....	-	-	-	15	+	-	-	-
R16.....	+	-	-	16	±	-	-	-
R17.....	-	-	-	17	+	-	-	-
R18.....	±	±	±	18	+	-	-	-
R19.....	-	-	-	19	±	-	-	-
*R21.....	+	+	+	20	±	-	-	-
R22.....	±	±	±	21	+	-	-	+
R23.....	-	-	-	22	±	-	-	-
R24.....	-	-	-	23	±	-	-	-
R25.....	-	-	-	24	±	-	-	-
RN1.....	-	-	-	25	-	-	-	-
RN2.....	+	-	-	1	-	-	-	-
*RN3.....	±	-	-	*2	+	-	-	-
RN4.....	+	+	+	*3	+	+	+	+
				4	+	+	+	+

[illegible]

matic subjects in New York City under continuous clinical observation, studies of the throat flora have been conducted. Hemolytic streptococcus in most instances appeared in the pharynx from 1 to 3 weeks before the onset of the rheumatic attack. These organisms have been investigated with the usual types of bacteriological tests and in addition, have been classified serologically according to Lancefield's technique. The results have demonstrated that the organisms were not of a single type, but fell into six antigenic groups. The majority of the freshly isolated strains tested were strong toxin producers. The organisms producing the strongest toxin were cultures from the patients who developed extremely intense rheumatism. About 70 per cent of these toxins were neutralized by a monovalent streptococcus antiserum.

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# STUDIES ON THE RELATIONSHIP OF STREPTOCOCCUS HEMOLYTICUS TO THE RHEUMATIC PROCESS

## III. OBSERVATIONS ON THE IMMUNOLOGICAL RESPONSES OF RHEU- MATIC SUBJECTS TO HEMOLYTIC STREPTOCOCCUS

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There are three phases to the rheumatic attack: first, a brief illness of respiratory infection with hemolytic streptococcus; second, a quiescent interval of approximately 10 days; third, the period of clinical manifestations of rheumatic fever. The relationship of a throat infection to the development of the rheumatic attack was described in the first paper of this series. In each of the three groups being studied, nurses, convalescent rheumatic patients and ambulatory rheumatic subjects, there was a well defined quiescent interval between the subsidence of the local infection and the onset of the rheumatic attack. The observations in 1930-31 illustrate these findings.<sup>1</sup> In the case of each student nurse, the quiescent period was at least 3 weeks; in the case of convalescent rheumatic patients at The Pelham Home, it varied from 5 to 27 days, with an average of 12 days; among the ambulatory rheumatic subjects there was usually a similar lag; however, in a few instances severe rheumatic phenomena followed immediately after a "sore throat" subsided.

The precise date of onset of rheumatism could not be judged with accuracy, and for this reason it has not been possible to determine the exact length of the quiescent interval. In the case of certain severe local infections and of a few tonsillectomies, almost immediate rheumatic attacks were observed. The usual finding was a definite period

<sup>1</sup> Haig-Brown (1) in 1886 and more recently Hector (2), Campbell and Warner (3) and Schlesinger (4) have also observed the constant occurrence of this quiescent interval.

of quiescence, which varied from a few days to a few weeks. The observations in this study are adequately described by the words of Haig-Brown (1) written nearly half a century ago: "perhaps a month, more commonly 10 days or simultaneously."

*Observations in The Pelham Home*

Throat infections	Interval	Onset of rheumatic attack	Throat infections	Interval	Onset of rheumatic attack
	days			days	
Apr. 21-22	14	May 6	Feb. 22-25	8	Mar. 6
Mar. 1-3	9	Mar. 12	June 17-20	22	July 12
Sept. 23-25	5	Oct. 1	Jan. 24-26	10	Feb. 6
Jan. 25-27	5	Feb. 2	Mar. 23-24	27	Apr. 21

*Observations on Ambulatory Rheumatic Subjects*

Throat infections	Interval	Rheumatic attack	Throat infections	Interval	Rheumatic attack
	days			days	
Feb. 21, 22, 23	10	Mar. 7	Apr. 16-18	8	Apr. 27
May 1	10	May 10	Jan. 25	3	Jan. 28
Mar. 1, 2, 3, 4	8	Mar. 12	Feb. 21-25	12	Mar. 9
Feb. 4	3	Feb. 7	Nov. 29	5	Dec. 3
Jan. 27, 28	7	" 5	" 1	14	Nov. 14
" 15	5	Jan. 21	Jan. 18	20	Feb. 7
Feb. 12-16	17	Mar. 5	Feb. 22	10	Mar. 2
" 14-16	17	" 3	" 13	7	Feb. 20

The occurrence of a 10 day period of quiescence between the subsidence of infection and the onset of rheumatism suggests that the rheumatic attack begins when the immune response is at its height. This observation has led to the conception that the development of rheumatic fever may be associated with the immunity mechanism of certain individuals for handling products of hemolytic streptococcus. In order to investigate the subsidence of the local infection and the development of rheumatism, sera have been examined for the presence of antibodies to hemolytic streptococcus. Four types of reactions were selected for investigation: agglutination; fixation of complement; occurrence of precipitins; development of antistreptolysin. The

methods employed and the findings for the 4 year period 1928-32 are as follows:

### *A. Agglutination Reactions*

During the early part of this study the blood sera of a number of acutely ill rheumatic patients were tested for the presence of agglutinins to *Streptococcus viridans*. Although agglutinins were present in high titer in patients with bacterial endocarditis, there was no instance in which this was true of the sera of patients with acute rheumatism.

The sera of five groups of individuals were tested for the presence of agglutinins to hemolytic streptococcus against strains of hemolytic streptococcus: No. C17 (rheumatic fever throat strain), hemolytic streptococcus Nos. NY5 and 273 (scarlet fever strains). The sera tested were obtained from the following groups: individuals with proven hemolytic streptococcus infections (scarlet fever, tonsillitis, erysipelas); acutely ill rheumatic patients; quiescent rheumatic subjects; patients with lobar pneumonia; normal, healthy individuals as controls.

The sera<sup>2</sup> of the patients in the first two groups in most instances agglutinated one or all of the organisms to a titer 1:10 to 1:40. In a few instances agglutination occurred in titer 1:80. In only one instance was agglutination observed in titer 1:160. The sera of the pneumonia patients, quiescent rheumatic subjects and controls usually failed to agglutinate entirely or agglutinated to a titer no higher than 1:40. These tests demonstrated that the sera of acutely ill rheumatic subjects resemble in their agglutination titer the sera of patients recovering from streptococcus infection. Because of their low titer this method of study was regarded as unsuitable.

### *B. Complement Fixation Reactions*

Complement fixation tests were performed with three antigens—hemolytic streptococcus carbohydrate, scarlet fever streptococcus toxin, scarlet fever streptococcus nucleoprotein. With the carbohydrate fractions and toxins no positive reactions were obtained, and these procedures were discontinued. The protein fractions, however,

<sup>2</sup> Some of these tests were done through the kindness of Dr. M. H. Dawson.



gave reactions and were made up in the following dilution of milligrams of protein:

1. No. 273 —1:1,000; 1:10,000; 1:15,000; 1:20,000; 1:25,000; 1:50,000
2. " C15DIII—1:1,000; 1:10,000; 1:15,000; 1:20,000; 1:25,000; 1:50,000
3. " C17D —1:1,000; 1:10,000; 1:15,000; 1:20,000; 1:25,000; 1:50,000

The fractions tested showed great differences in their antigenic strength. Fraction C8D gave only negative reactions and Fraction C15DIII gave reactions only about one-fifth as strong as Fraction 273, of the same dilution. This is illustrated in the following table. The reactions with Protein 273 were consistently more intense than those with C15D—illustrated by two rheumatic patients, S and D.

Dilutions of antigen.....	1:10,000	1:20,000	1:40,000	1:50,000	1:100,000
S (C15D).....	++++	++±	—	—	—
S (273).....	++++	+++++	+++	++±	++
D (C15D).....	++++	+	—	—	—
D (273).....	++++	+++	++	—	—

The technique employed for these tests was the method customarily employed in the Wassermann reactions. The tubes together with a positive and negative serum control as well as antigen controls consisting of 0.4, 0.3, 0.2, 0.1 cc. amounts were water bathed at 37.5°C. for 1 hour, after which each tube received 0.2 cc. of sensitized 5 per cent sheep cells and was again incubated at 37.5°C. Readings were made as soon as the antigen and serum controls cleared and were interpreted as follows:

Human serum reactions	0.02 cc.		0.01 cc.	
	No	hemolysis	No	hemolysis
++++	"	"	Slight	"
+++	"	"	Complete	"
++	Slight	"	"	"
+	Complete	"	"	"
Negative				

The patients whose sera were tested included a control group believed to be non-rheumatic; a control group of student nurses, some known to be in good health, others known to have acute hemolytic streptococcus pharyngitis; rheumatic subjects believed to be quiescent, and patients with acute rheumatic fever. The determinations are recorded in Table I.

TABLE I

*Complement Fixation Reactions*  
*Antigen Nucleoprotein C15DIII*

Dilutions of antigen.....	1:1,000	1:10,000	1:15,000	1:20,000	1:25,000	1:30,000	1:35,000	1:40,000
Control group, non-rheumatic; spring months								
T.....	-	-	-	-	-	-	-	-
Be.....	-	+	-	-	-	-	-	-
Bo.....	-	±	-	-	-	-	-	-
G.....	-	-	-	-	-	-	-	-
N.....	-	-	-	-	-	-	-	-
P.....	-	-	-	-	-	-	-	-
Nurses, normal and recovering from hemolytic streptococcus pharyngitis; spring months								
Br., normal.....	-	-	-	-	-	-	-	-
L. ".....	++	-	-	-	-	-	-	-
S. ".....	+++	-	-	-	-	-	-	-
C.,* pharyngitis.....	+++	++++	++	++	+	-	-	-
T. ".....	-	-	-	-	-	-	-	-
F. ".....	++	+	-	-	-	-	-	-
H. ".....	+	-	-	-	-	-	-	-
Rheumatic subjects clinically quiescent; spring months†								
Fi.....	-	-	-	-	-	-	-	-
C.....	-	-	-	-	-	-	-	-
Fa.....	++++	++++	++++	+++	+++	+	-	-
W. H.....	-	-	-	-	-	-	-	-
Wa.....	-	-	-	-	-	-	-	-
Ma.....	-	++	-	-	-	-	-	-
W.....	-	-	-	-	-	-	-	-
La.....	-	-	-	-	-	-	-	-
Ku.....	-	+	-	-	-	-	-	-
Active rheumatism; spring months								
Ma.....	-	±	+	±	-	-	-	-
Gi.....	-	+	-	-	-	-	-	-
Ga.....	-	+++	+	±	-	-	-	-
Fe.....	-	++++	-	-	-	-	-	-
Fa.....	-	+	-	-	-	-	-	-
Pa.....	-	++++	-	-	-	-	-	-
T.....	-	+	-	-	-	-	-	-
Br.....	-	+	-	-	-	-	-	-
H.....	-	+	-	-	-	-	-	-

\* Cand T had scarlet fever. † Serum examined during the fall showed no strong reactions.

As is indicated in Table I, the complement fixation reaction appeared extremely sensitive for the determination of antibody. Because of the variability in hemolytic systems the method was not found satisfactory for sharp interpretation. In certain instances, the strongest reactions were detected among rheumatic patients clinically quiescent; while in fulminating attacks the reaction was frequently less striking. The sera of non-rheumatic individuals infected by hemolytic streptococcus gave a positive fixation at a dilution 1:1,000 in most instances, and the serum of each acutely ill rheumatic subject gave a positive complement fixation in 1:10,000 dilution of the antigen. This type of immunity response suggests a recent infection with *Streptococcus hemolyticus*.

The results of the complement fixation tests are as follows: The majority of the control sera were negative. The sera of individuals convalescing from sore throat or scarlet fever were positive. The sera of some rheumatic subjects without clinical symptoms showed strongly positive reactions in the spring. The sera of all rheumatic subjects during the attack showed a positive reaction, but in some instances this reaction was not so strong as in the sera of individuals who were symptom-free.

### C. *Precipitin Reactions*

To investigate further the possible relationship of the rheumatic attack to infection with *Streptococcus hemolyticus*, the occurrence of precipitins to hemolytic streptococcus fractions has been studied in detail over a long period of time. That these reactions are not entirely specific has been demonstrated by the work of Lancefield (5) which indicates an antigenic relationship of the proteins of streptococcus to those of pneumococcus, staphylococcus and other organisms.

The materials used as precipitinogen consisted of the following hemolytic streptococcus substances: toxins, carbohydrates and proteins. The toxins were tested for precipitation in several dilutions and the carbohydrate fraction in concentrations 1:25,000, 1:100,000, and 1:500,000. Reactions with the former were uniformly negative and with the latter rarely positive. Their use was discontinued. The protein fractions of *Streptococcus* 273 previously used in skin testing (6)<sup>3</sup> and protein fractions recently prepared by Dr. Michael Heidelberger (7) have been employed throughout the study. The D fractions were obtained after acidification of the cells at pH 6.5. The K fractions and 273 were obtained at pH 11 to 13 after removal of the fractions between pH 6.5 and pH 11. In general the dilution used was milligrams of protein 1:2,000.

The technique of precipitin tests was as follows: To 0.2 cc. each of fresh human sera, was added an equal quantity of nucleoprotein fraction. (It is essential to have both clear sera and protein solutions.) After mixture each tube was immediately and thoroughly shaken. Readings were taken at various intervals, the first after 20 minutes at room temperature, the second after 2 hours in 37.5°C. water and the third after overnight refrigeration.

The development of precipitins was first studied among a class of 50 student nurses. The antigen used was hemolytic streptococcus Frac-

<sup>3</sup> Coburn (6), p. 226.

tion C15D, dilution 1:2,000. In September, 1930, all of the individuals were tested for the presence of precipitins in their blood and none were found. During the spring months the sera of the same individuals were again tested, and the results are summarized in Table II.

TABLE II

*A Study of Precipitin Reactions to Hemolytic Streptococcus Fractions among a Class of 50 Nurses*

Patient	Condition	Fall, 1930	Spring, 1931
<i>Group A</i>			
B.....	Normal health		— — —
L.....	" "	— — —	— — —
M.....	" "	— — —	— — —
S.....	" "	— — —	— — —
<i>Group B</i>			
S.....	Normal health		
	*Hemolytic <i>Streptococcus pharyngitis</i>	— — —	— — —
W.....	Normal health		
	Hemolytic <i>Streptococcus pharyngitis</i>	— — —	+ + ± + + +
H.*.....	" " "		— — —
Bix.....	" " "		— — + ±
Bil.....	" " "		— + +
F.....	" " "		— — ±
<i>Group C</i>			
T.....	Normal health		
	Scarlet fever	— — —	— — —
C.....	Normal health		
	Scarlet fever	— — —	— ± ±
M.....	" "		— — —
<i>Group D</i>			
Cr.....	Normal health		
	Acute hemolytic streptococcus infection followed by rheumatic fever	— — —	+ + ± + + +
Me.....	Normal health		
	†Acute hemolytic streptococcus infection followed by rheumatic fever	— — —	— — ±
Sm.....	Normal health		
	Acute hemolytic streptococcus infection followed by rheumatic fever	— — —	+ + ± + + +

These readings are expressed: one, immediately; second, at 2 hours at 37°C. and the last, overnight in ice box. They represent uncentrifuged findings. + + + + = complete disc at bottom of tube with clear supernatant. + + + = flocculated sediment. + + = no sediment; granules or floccules in suspension. + = clouding with whirl. ± = clouding with no whirl.

Intermediate readings were also made, such as, + + + ± = incomplete disc. + + ± = flocculated sediment with some large floccules still in suspension. + ± = few granules or floccules in suspension with slight clouding of supernatant.

\* At onset. † Erythema marginatum—no arthritis.

From Table II, it is seen that Group A escaped infection and their sera remained negative for precipitins; Group B developed hemolytic streptococcus pharyngitis and precipitins appeared in some instances during convalescence; Group C contracted scarlet fever and precipitins were not detected during the acute stage of the illness; Group D contracted hemolytic streptococcus pharyngitis and developed rheumatic fever. The appearance of precipitins to hemolytic streptococcus protein fractions in their sera was striking.

A second study was made to determine the relationship of the development of precipitins for hemolytic streptococcus fractions to activity of the process in rheumatic subjects. At the end of the summer, the rheumatic process appeared quiescent in nearly every individual. During the early fall, two of the group contracted pharyngitis and developed severe rheumatic attacks. In the late winter months, most of the patients were probably exposed to infection. Some escaped symptoms of upper respiratory disease and the rheumatic process appeared quiescent. Others contracted throat infections. Most of these developed severe rheumatic attacks; a few appeared to escape. With the exception of these few individuals, the results of the precipitin tests are presented in Table III.

From Table III it is seen that in the sera of quiescent rheumatic subjects precipitins were not detected in the fall. Sera from the same individuals, clinically quiescent in the spring, gave in most instances negative or weakly positive reactions. In contrast, the sera of rheumatic patients acutely ill in the fall or in the spring months in almost each instance gave a strong precipitin reaction to protein fractions of hemolytic streptococcus. This occurred only in the individuals who developed acute rheumatism. It was pointed out in the first paper of this series that in certain instances following hemolytic streptococcus infection the rheumatic subject escapes a definite attack. The sera of these patients have been studied at frequent intervals during the 4 weeks following infection. In none were precipitins detectable.

In order to correlate the clinical signs of activity of the disease process with the content of precipitins to fractions of hemolytic streptococcus in the sera of rheumatic subjects, a few individuals have been studied closely. Patient Ker. during a severe rheumatic attack in December, 1930, had strong precipitins in her serum. These dis-

appeared with clinical improvement and reappeared again during another intense recrudescence. Individual Kor. with a fulminating rheumatic attack in November, 1930, likewise showed a high concentration of precipitins. Signs of mild rheumatic activity persisted in

TABLE III  
*A Study of the Relationship of the Precipitin Reaction to Quiescence and Activity of the Rheumatic Process*

Patient	Fall, 1930		Spring, 1931	
	273	C15D	273	C15D
Group A—apparently quiescent				
C.....	— — —	— — —	— — —	— — —
H.....	— — —	— — —	— — —	— — —
Ki.....	— — —	— — —	— — —	— — —
La.....	— — —	— — —	— — —	— — —
Lu.....	— — —	— — —	— — —	— — —
Ma.....	— — —	— — —	— — —	— — —
Ri.....	— — —	— — —	— — —	— — —
Ru.....	— — —	— — —	— — —	— — —
Wa.....	— — —	— — —	— — —	— — —
Wi.....	— — —	— — —	— — —	— — —
Fa.....	— — —	— — —	— — —	— — —
Group B—active rheumatism				
Ker.....	+	+	+	+
Kor.....	+	+	+	+
Kel.*.....	+	+	+	+
Cl.†.....	+	+	+	+
Fa.....	+	+	+	+
Br.....	+	+	+	+
T.....	+	+	+	+
O.....	+	+	+	+
P.....	+	+	+	+
S.....	+	+	+	+
F.....	+	+	+	+
G.....	+	+	+	+
Ga.....	+	+	+	+
Gi.....	+	+	+	+

\* Negative 1 month before attack.

† Negative 4 months before attack.

this patient for 6 months. The precipitins did not disappear during this period, increased during a slight recrudescence in March, 1931, and finally were no longer detectable when the disease seemed quiescent. These findings are presented in Table IV, a.

In addition to the patients in Table IV, a, showing the presence of

TABLE IV, *a*

*Changes in the Precipitin Reaction with Fluctuations in Activity of the Rheumatic Process*

Patient	Protein fraction, dilution 1:2,000			
	C7L	C8D	273	C15D
<i>Ker.</i>				
During attack, Nov., 1930.....	+ + +	++ + ++	- + +++	
During convalescence, Jan., 1931.....		- - -	- - -	
Feb., 1931.....		- - -	- - -	
During recrudescence, Apr., 1931.....				- - ±
During attack, May, 1931.....				± + +
During quiescence, Feb., 1932.....		- - -(-)*	- - -(-)	- - -(+)
<i>Kor.</i>				
During attack, Nov. 1, 1930.....	++ + -	++ + -	+ + +++	
Nov. 30, 1930.....	- - -	- - -		- ± ±
During convalescence, Dec., 1930.....	- - -	- - ±	± - -	
During slight flare up, Mar., 1931.....		- - +	- - +	- + +±
During quiescence, Feb., 1932.....		- - -(-)	- - -(-)	- - -(-)

\* In addition to the customary readings made immediately, after 2 hours' incubation and after standing overnight in the ice box, a fourth determination has been recorded when indicated. This is expressed in parentheses and represents the findings after 10 minutes' centrifugalization at moderate speed.

TABLE IV, *b*

*Changes in the Precipitin Reaction in Relation to the Clinical Course of the Rheumatic Attack*

Patient	Date						
	Dec. 3	Dec. 24	Dec. 28	Jan. 4	Jan. 11	Jan. 19	Feb. 9
<i>Maz.</i>							
Condition.....	Sick	Sick	Very sick	Sick	Recovering		
Reactions to 1:2,000 dilution of Fraction C17D.....	+	+	+ ±	++	+	±	±
Fraction C17K.....	+	+	+ ±	++	+	±	±
	Jan. 3	Jan. 6	Jan. 10	Jan. 15	Jan. 27		
<i>Man.</i>							
Condition.....	Sick	Sick	Very sick		Moribund		
Reactions to 1:2,000 dilution of Fraction C17D.....	±(+)*	-(+)	+(++)	+	+ ±		
Fraction C17E.....	+(++)	+(+)	+(+++)	+	+ ±		

\* Parentheses indicate readings after centrifugation, single determinations represent 18 hour uncentrifuged readings.

precipitins during acute rheumatism, absence during recovery and re-appearance during recrudescence, the sera of other individuals were examined at frequent intervals during a single, severe attack of rheumatic fever. The findings in two of these are presented in Table IV, *b*. Patient Maz. experienced a fulminating rheumatic attack and recovered; Patient Man. died 3 weeks after the onset of intense rheumatism.

TABLE V

*A Study of the Precipitin Reaction to Streptococcus Protein Fractions*

Patient	273	C15D
Scarlet fever		
Mc.*.....	+ + +	+ + +
Br.....	- + ±	- - -
R.....	- ± -	- - -
H.....	± + +	- - -
L.....	- ± -	- - -
R.....	± - -	- - -
Lobar pneumonia (pneumococcus)		
W.-Pn. IV.....	- + +	- - -
B.-Pn. III.....	- - ±	- - ±
Bo.-Pn. II.....	- + +	- - +
Gi.-Pn. IV.....	- + +	- - +
Sh.-Pn. IV.....	- - -	- - -
Erysipelas and mastoiditis		
T.....	- - ±	- - ±

\* Mc. developed rheumatic heart disease.

From Table IV, *b*, it is seen that with recovery, the precipitin content diminished in the serum of Patient Maz. In contrast, the content of precipitins to hemolytic streptococcus protein fraction increased in the serum of Patient Man. to the day of death. These were both examples of intense rheumatism. In the less severe attacks, the precipitin content of the sera has been found to change more rapidly, remaining at a high level for only a few days. There is a close parallelism between intensity of the rheumatic process and the strength of the precipitin reactions.

For purposes of comparison, a study was made of the content of precipitins in the sera of patients with a variety of respiratory infec-



tions. Determinations were made during the course of lobar pneumonia, during the 3rd week of scarlet fever and during recovery from erysipelas. The findings are presented in Table V.

From Table V it is seen that weak precipitin reactions occurred in the sera of patients with respiratory infections. The only strong per-

TABLE VI, a

*Precipitin Reactions to Hemolytic Streptococcus Fractions in Quiescent Rheumatic Subjects Inoculated with T.A.B. Vaccine*

Patient	Fraction 273	Fraction 17D	Fraction 17K	Control
Before vaccination with <i>B. typhosus</i> and <i>paratyphosus</i> A and B				
Br.....	- - - (-)	- - - (++)	- - - (+)	- - - (-)
By.....	- - - (-)	- - - (-)	- - - (-)	- - - (-)
C.....	- - - (-)	- - - (-)	- - - (-)	- - - (-)
H.....	- - - (-)	- - - (-)	- - - (-)	- - - (-)
M.....	- - - (-)	- - - (-)	- - - (-)	- - - (-)
Mo.....	- - - (-)	- - - (-)	- - - (-)	- - - (-)
1 and 3 wks. after vaccination				
Br.....	- - - (-)	- - - (+±)	- - ± (+)	- - - (-)
By.....	- - - (-)	- - - (+±)	- - - (-)	- - - (-)
C.....	- - - (-)	- - - (-)	- - - (-)	- - - (-)
H.....	- - - (-)	- - - (-)	- - - (-)	- - - (-)
M.....	- - - (-)	- - - (-)	- - + (-)	- - - (-)
Mo.....	- - - (-)	- - - (-)	- - - (-)	- - - (-)
R.....	- - - (-)	- - - (-)	- - - (-)	- - - (-)

TABLE VI, b

*Precipitin Reactions in a Rheumatic Subject with Pneumococcus Type II Pneumonia*

Patient Cr.	Fraction 17D	Fraction 17K	Control
1. Before Nov. 1, 1930.....	- - - (±)	- - - (±)	- - - (±)
2. Onset Jan. 22, 1932.....	- - ± (+)	- - - (±)	- - - (±)
3. During Jan. 27, 1932.....	- - + (+±)	- - + (±)	- - - (±)
4. Convalescence Feb. 8, 1932.....	- - ±	- - ±	- - ±
5. Recovery Feb. 22, 1932, 4 wks. after onset.....	- - - (±)	- - - (-)	- - - (-)

sistent precipitin reaction among these patients was in an individual with scarlet fever which terminated in rheumatic heart disease.

As a control measure, studies were made to determine the influence of a reaction to heterologous bacterial proteins on the formation of precipitins to hemolytic streptococcus products. First, ten quiescent

rheumatic patients, who were under close observation at The Pelham Home, were given one subcutaneous dose of T.A.B. vaccine (*B. typhosus* 1 billion, Para A and Para B  $\frac{1}{2}$  billion each). This was followed by a sharp reaction in almost each instance. The sera were tested before inoculation, and at the end of 1 and 3 weeks. Second, a few rheumatic subjects contracted pneumococcus lobar pneumonia. Their sera were tested during the disease and in convalescence. The findings are presented in Tables VI, *a*, and VI, *b*.

As shown in Tables VI, *a*, and V, *b*, the precipitin content of the sera was not influenced by a non-specific reaction. A few rheumatic subjects with pneumonia showed the presence of weak precipitins during pneumococcus infection and their disappearance in convalescence.

### *Summary of Precipitin Tests*

The results may be summarized as follows:

In the sera of 150 apparently healthy rheumatic subjects in the fall of 1930, only two individuals were found to have precipitins for hemolytic streptococcus protein fractions. In the sera of 50 healthy student nurses entering training in the fall of 1930, no precipitins were detected. In the sera of patients with lobar pneumonia, the formation of precipitins to hemolytic streptococcus fractions was slight, definite but not persistent. In the sera of twenty patients during the acute stages and convalescence from scarlet fever or erysipelas, marked precipitin formation was detected in one individual. She was the member of a rheumatic family and developed rheumatic carditis. In the sera of twenty student nurses who contracted hemolytic streptococcus throat infections in the spring of 1931, precipitins were not detected during the acute illness but appeared 4 weeks later in slight concentration in most individuals. In four instances the appearance of precipitins was marked; three of these individuals developed rheumatic fever. In the sera of ten rheumatic subjects given prophylactic vaccination with typhoid and paratyphoid vaccine, there was no development of precipitins to the protein fractions of hemolytic streptococcus. One rheumatic subject who contracted *Pneumococcus* Type II pneumonia developed precipitins to protein fractions of a streptococcus but they disappeared with complete resolution in the lung and did not reappear within 4 weeks. In the sera of ten patients with

acute rheumatism in the fall months of 1930 and 1931, precipitins were detected in each serum.<sup>4</sup> In the sera of 50 patients with fulminating rheumatism in the spring months of 1928-31, precipitins were detected in all except two instances. In the sera of six rheumatic subjects who appeared to escape attacks following hemolytic streptococcus infection, precipitins were not detected. In the sera of rheumatic subjects without demonstrable precipitins in the fall of 1930, fourteen manifested rheumatic attacks in the spring and all developed precipitins. Of thirty whose disease appeared to remain quiescent in the spring, five developed precipitins. This was marked in only one instance. In the sera of ten individuals with acute rheumatism, the concentration of precipitins became more marked as the activity of the process heightened. With subsidence, precipitins disappeared. They returned during recrudescence. In one fatal, fulminating attack which perhaps represented the onset of the disease in a rheumatic baby, the precipitin titer rose during the fortnight's illness and was marked on the day of death.

#### *D. The Development of Antistreptolysin*

Through the kindness of Dr. E. W. Todd of The Belmont Laboratories, Sutton, England, the development of another antibody has been studied in the patients described in the first paper of this series. While working with streptococcal hemolysin, Todd (9) observed that antihemolysin was not formed in animals immunized to Klebs-Löffler bacillus, pneumococcus, hemolytic staphylococcus, non-hemolytic streptococcus or other infectious agents, but only in animals immunized to *Streptococcus hemolyticus*. In conjunction with Dr. Todd, the antihemolysin titers of the sera previously examined for precipitins have been determined. Some of these findings are being reported elsewhere (10) in units of antihemolysin. In the present paper, the antistreptolysin titers are recorded as the volume of the patient's serum required to neutralize  $2\frac{1}{2}$  minimal hemolytic doses of yeast extract streptolysin (M.H.D.). The titers are therefore expressed as fractions of 1.0 cc., and N.D. is used to designate neutralizing

<sup>4</sup> These sera were tested with pneumococcus "C" substance. Positive reactions were obtained only during the febrile period. This phenomenon was identical with that previously described by Tillett and Francis (8).

dose. The serum of Patient Ker. is used as a control throughout the present study.

The sera of three groups of non-rheumatic subjects were tested first.<sup>5</sup> These consisted of normal individuals in good health; patients convalescing from hemolytic staphylococcus or tuberculous bone infections, and individuals recovering from hemolytic streptococcus infection. The findings are presented in Table VII.

TABLE VII

*A Study of the Antistreptolysin Content of Sera from Rheumatic Subjects*

A. Normal individuals					
		cc.			cc.
Wh....	Good health	N.D. = 0.05	Wa....	Good health	N.D. = 0.02
Wo....	" "	" = 0.05	Do....	" "	" = 0.02
Ba....	" "	" = 0.02	Mo....	" "	" = 0.02
Ha....	" "	" = 0.1	Io....	" "	" = 0.02
To....	" "	" = 0.3	Wi....	" "	" = 0.02
Co....	" "	" = 0.01			
B. Patients convalescing from infections other than hemolytic streptococcus					
		cc.			cc.
Gr....	Osteomyelitis	N.D. = 0.03	Ga....	Tuberculosis of hip	N.D. = 0.02
Sh....	" "	" = 0.02	Ba....	Poliomyelitis	" = 0.07
Go....	Hemolytic staphylococcus abscess	" = 0.02	Ta....	Psoas abscess	" = 0.02
Bn....	" "	" = 0.02	Kl....	Tuberculous spine	" = 0.02
Ca....	Tuberculosis of hip	" = 0.02	Pe....	" "	" = 0.009
C. Patients convalescing from hemolytic streptococcus infections					
		cc.			cc.
Ca....	Scarlet fever,* 1st wk.	N.D. = 0.01	In....	Scarlet fever, 3rd wk.	N.D. = 0.004
Ro....	" " 1st "	" = 0.006	Mc....	" " 4th "	" = 0.005
Br....	" " 3rd "	" = 0.006	He....	" " 8th "	" = 0.003
Hi....	" " 3rd "	" = 0.006	Tu....	Erysipelas, 3rd "	" = 0.0003

\* These sera were obtained through the kindness of the Willard Parker Hospital.

† Mc. developed rheumatic fever.

Table VII shows that the sera of individuals and patients with tuberculous or hemolytic staphylococcus infection had a low content of antistreptolysin. In contrast, the serum of patients with hemolytic streptococcus infection was high in almost each instance.

<sup>5</sup> All of these antistreptolysin determinations were made by Dr. E. W. Todd, Belmont Laboratories, Sutton, Surrey, England. Through his generosity they are presented here.

acute rheumatism in the fall months of 1930 and 1931, precipitins were detected in each serum.<sup>4</sup> In the sera of 50 patients with fulminating rheumatism in the spring months of 1928-31, precipitins were detected in all except two instances. In the sera of six rheumatic subjects who appeared to escape attacks following hemolytic streptococcus infection, precipitins were not detected. In the sera of rheumatic subjects without demonstrable precipitins in the fall of 1930, fourteen manifested rheumatic attacks in the spring and all developed precipitins. Of thirty whose disease appeared to remain quiescent in the spring, five developed precipitins. This was marked in only one instance. In the sera of ten individuals with acute rheumatism, the concentration of precipitins became more marked as the activity of the process heightened. With subsidence, precipitins disappeared. They returned during recrudescence. In one fatal, fulminating attack which perhaps represented the onset of the disease in a rheumatic baby, the precipitin titer rose during the fortnight's illness and was marked on the day of death.

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dose. The serum of Patient Ker. is used as a control throughout the present study.

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		cc.			cc.
Wh....	Good health	N.D. = 0.05	Wa....	Good health	N.D. = 0.02
Wo....	" "	" = 0.05	Do....	" "	" = 0.02
Ba....	" "	" = 0.02	Mo....	" "	" = 0.02
Ha....	" "	" = 0.1	Io....	" "	" = 0.02
To....	" "	" = 0.3	Wi....	" "	" = 0.02
Co....	" "	" = 0.01			
B. Patients convalescing from infections other than hemolytic streptococcus					
		cc.			cc.
Gr....	Osteomyelitis	N.D. = 0.03	Ga....	Tuberculosis of hip	N.D. = 0.02
Sh....	" "	" = 0.02	Ba....	Poliomyelitis	" = 0.07
Go....	Hemolytic staphylococcus abscess	" = 0.02	Ta....	Psoas abscess	" = 0.02
Bn....	" "	" = 0.02	Kl....	Tuberculous spine	" = 0.02
Ca....	Tuberculosis of hip	" = 0.02	Pe....	" "	" = 0.009
C. Patients convalescing from hemolytic streptococcus infections					
		cc.			cc.
Ca....	Scarlet fever,* 1st wk.	N.D. = 0.01	In....	Scarlet fever, 3rd wk.	N.D. = 0.004
Ro....	" " 1st "	" = 0.006	†Mc....	" " 4th "	" = 0.005
Br....	" " 3rd "	" = 0.006	He....	" " 8th "	" = 0.003
Hl....	" " 3rd "	" = 0.006	Tu....	Erysipelas, 3rd "	" = 0.0003

\* These sera were obtained through the kindness of the Willard Parker Hospital.

† Mc. developed rheumatic fever.

Table VII shows that the sera of individuals and patients with tuberculous or hemolytic staphylococcus infection had a low content of antistreptolysin. In contrast, the serum of patients with hemolytic streptococcus infection was high in almost each instance.

<sup>5</sup> All of these antistreptolysin determinations were made by Dr. E. W. Todd, Belmont Laboratories, Sutton, Surrey, England. Through his generosity they are presented here.

A second series of observations was made on a group of normal student nurses to observe the effect of streptococcus infections on the antistreptolysin titer. On admission to the Training School in September, 1930, while apparently free of hemolytic streptococcus and in good health, their sera showed a normal titer (except in two instances). During the month of April, 1931, sera were again obtained. The individuals were divided into three groups: those exposed to infection but in good health; those acutely ill with scarlet fever; those

TABLE VIII

*The Influence of Hemolytic Streptococcus Infection on the Antistreptolysin Titer of Student Nurses under Close Observation Clinically and Bacteriologically*

Apr., 1931	
Group A. In good health but exposed to infection	
Patient L.....	cc. N.D. = 0.02
" B.....	" = 0.03
" S.....	" = 0.02
Group B. At onset of scarlatina	
Patient T.....	cc. N.D. = 0.07
" C.....	" = 0.02
Group C. During convalescence from hemolytic streptococcus tonsillitis	
Patient Fan.....	cc. N.D. = 0.007
" B.....	" = 0.003
" H.....	" = 0.005
" S.....	" = 0.007

convalescing from hemolytic streptococcus pharyngitis. The results are summarized in Table VIII.

Group A, the individuals who remained in good health, although exposed to infection, showed no change in titer; Group B, the individuals acutely ill with hemolytic streptococcus throat infections, showed no change in titer; Group C, the individuals convalescing from hemolytic streptococcus throat infections, showed a marked rise in antistreptolysin content. These determinations indicate that there occurs normally a rise in antistreptolysin content of the serum during convalescence from hemolytic streptococcus infection.

For purposes of control, two studies were made to test the effect of non-specific stimulation with a heterologous antigen. First, the sera of ten children convalescing from rheumatic fever were tested before and after T.A.B. subcutaneous inoculation. Second, the serum of a patient with rheumatic heart disease was tested before, during and after *Pneumococcus* Type II pneumonia. The results are presented in Tables IX, *a*, and IX, *b*.

TABLE IX, *a*

*Antistreptolysin Titers of Convalescent Rheumatic Patients before and 1 and 3 Weeks after Typhoid Vaccination*

Patient		Be- fore	1 wk. after	3 wks. after	Patient		Be- fore	1 wk. after	3 wks. after
		cc.	cc.	cc.			cc.	cc.	cc.
Br.....	N.D. =	0.009	0.009	0.02	Kr.....	N.D. =	0.006	0.006	
By.....	" =	0.02	0.02		Me.....	" =	0.009	0.009	
Cu.....	" =	0.02	0.02		Mo.....	" =	0.004	0.02	0.03
Ga.....	" =	0.02	0.02		Po.....	" =	0.02	0.02	0.02
Ha.*.....	" =	0.01	0.01		Ra.....	" =	0.008	0.008	0.01

\*Ha. contracted hemolytic streptococcus pharyngitis 8 weeks later and in the 2nd week the N.D. rose to 0.008 cc.

TABLE IX, *b*

*Antistreptolysin Titers in a Rheumatic Subject with *Pneumococcus* Type II Pneumonia*

Patient Ch.		cc.
	Before pneumonia	N.D. = 0.004
	Onset of "	" = 0.006
	During "	" = 0.007
	Convalescing from pneumonia	" = 0.008

Table IX, *a*, shows that inoculation with T.A.B. vaccine did not increase the titer of antistreptolysin. The fall in titer of Patients Mo., Br. and Ra. is unexplained. Table IX, *b*, shows that during *Pneumococcus* Type II infection the antistreptolysin titer of a rheumatic subject did not rise but tended to fall to normal.

Because of the constant development of a high titer of antistreptolysin in individuals convalescing from infection with hemolytic streptococcus, a study was made of the content of this antibody in the sera of patients who, while under observation, contracted hemolytic strepto-



coccus infection and developed rheumatic fever. The individuals under investigation included children and adults. The sera were obtained during the 1st week of typical attacks of acute rheumatism. The findings are presented in Table X.

TABLE X

*A Study of the Antistreptolysin Content of Sera from Patients Developing Acute Rheumatism while Convalescing from Respiratory Infection with Hemolytic Streptococcus*

Patient			Patient		
		cc.			cc.
M.....	Acute rheumatic fever	N.D. = 0.007	Ro.....	Acute rheumatic fever	N.D. = 0.003
S.....	" " "	" = 0.003	Ri.....	" " "	" = 0.003
St.....	" " "	" = 0.007	N.....	" " "	" = 0.002
C.....	" " "	" = 0.007	O.....	" " "	" = 0.002
W.....	" " "	" = 0.004	Sh.....	" " "	" = 0.004

Table X shows the constantly high titer of antistreptolysin in the sera of patients with acute rheumatism following hemolytic streptococcus pharyngitis.

TABLE XI

*Antistreptolysin Titer of Patients Admitted to the Presbyterian Hospital Wards with Acute Rheumatic Fever from Spring, 1928, to Fall, 1931, and without Hemolytic Streptococcus in the Throat Flora*

Patient			Patient		
		cc.			cc.
K.....	Acute rheumatic fever	N.D. = 0.002	P.....	Acute rheumatic fever	N.D. = 0.003
M.....	" " "	" = 0.004	Co.....	" " "	" = 0.006
E.....	" " "	" = 0.004	Co.....	" " "	" = 0.008
E.....	" " "	" = 0.003	B.....	" " "	" = 0.008
S.....	" " "	" = 0.003	Cr.....	" " "	" = 0.006
S.....	" " "	" = 0.002	D.....	" " "	" = 0.006
L.....	" " "	" = 0.009	L.....	" " "	" = 0.004
C.....	" " "	" = 0.002	R.....	" " "	" = 0.005
M.....	" " "	" = 0.003	M.....	" " "	" = 0.004
Ch.....	" " "	" = 0.004	Sh.....	" " "	" = 0.006
Ch.....	" " "	" = 0.003	G.....	" " "	" = 0.008
P.....	" " "	" = 0.003			

Another series of observations were made on patients admitted to the Presbyterian Hospital with acute rheumatic fever and without

hemolytic streptococcus in the throat flora. The group included children and adults, in various seasons, between 1928 and 1931. The findings are presented in Table XI.

The determinations show that the sera of patients admitted to the hospital with acute rheumatic fever following respiratory infection had, irrespective of the bacteriology of the throat flora, a high titer of antistreptolysin.

A third series of determinations were made on the sera of patients with acute rheumatism who denied a preceding respiratory infection. The findings are presented in Table XII.

From Table XII it is seen that the sera of these individuals, like those of the patients known to have a respiratory infection prelimi-

TABLE XII  
*Antistreptolysin Titer of Patients with Acute Rheumatic Fever Who Denied  
Preceding Respiratory Infection*

Patient			Patient		
		cc.			cc.
F.....	Acute rheumatic fever	N.D. = 0.003	Dev.....	Acute rheumatic fever	N.D. = 0.002
K.....	" " "	" = 0.002	R.....	" " "	" = 0.002
H.....	" " "	" = 0.008	N.....	" " "	" = 0.002
Dem.....	" " "	" = 0.002			

nary to the rheumatic attack, contained a high titer of antistreptolysin. This was approximately the same as appears in convalescence from hemolytic streptococcus infection.

For purpose of comparison, a study was made of the antistreptolysin content of the sera of rheumatic subjects whose disease process appeared to be quiescent. The findings are presented in Table XIII.

Table XIII shows that in the majority of sera studied, the antistreptolysin content was normal. In a minority, the titer was elevated, but not to the same degree as observed in most patients with acute rheumatism.

During the acute rheumatic attack, the antistreptolysin titer was found to be high, and during inactivity of the disease process it appeared normal in most instances. To trace the changes in the antistreptolysin titer of rheumatic subjects, sera were examined at inter-

TABLE XIII

*Antistreptolysin Titer of Apparently Quiescent Rheumatic Subjects from Spring, 1928, to Fall, 1931*

Patient				Patient			
			cc.				cc.
Mul....	Jan. 14, 1932	Clinically inactive	N.D. = 0.02	Tre....	Jan. 7, 1931	Clinically inactive	N.D. = 0.03
Gill....	" 14, 1932	" "	" = 0.02	Wa....	Dec. 18, 1930	" "	" = 0.02
J. Jo....	" 14, 1932	" "	" = 0.007	Mo....	Feb. 4, 1932	" "	" = 0.02
J. Jo....	Oct. 8, 1930	" "	" = 0.01	Zi....	Nov. 1, 1930	" "	" = 0.05
Co....	June 10, 1931	" "	" = 0.01	Ro....	Jan. 23, 1932	" "	" = 0.03
Cou....	Jan. 14, 1931	" "	" = 0.03	Mah....	" 2, 1932	" "	" = 0.02
Ho....	Dec. 11, 1931	" "	" = 0.02	Bar....	" 7, 1931	" "	" = 0.03
Rom....	Jan. 7, 1932	" "	" = 0.02	La....	Oct. 4, 1930	" "	" = 0.006
Fl....	" 9, 1932	" "	" = 0.02	Mak....	" 4, 1930	" "	" = 0.009
Wy....	Oct. 1, 1930	" "	" = 0.02	Bus....	" 4, 1930	" "	" = 0.02
Sc....	June 10, 1931	" "	" = 0.02	Cer....	Jan. 7, 1931	" "	" = 0.02
Ba....	Oct. 1, 1930	" "	" = 0.03	Tri....	Dec. 17, 1930	" "	" = 0.04
D. Do..	Jan. 16, 1932	" "	" = 0.02	Len....	Jan. 28, 1932	" "	" = 0.03
Tr....	Nov. 1, 1930	" "	" = 0.009	Bea....	" 28, 1932	" "	" = 0.03
R. Ha..	Jan. 7, 1931	" "	" = 0.03	Hu....	" 29, 1932	" "	" = 0.009
Tol....	Dec. 13, 1930	" "	" = 0.005	Fe....	" 28, 1932	" "	" = 0.02

vals during health and disease. The findings are presented in Table XIV.

TABLE XIV

*Antistreptolysin Titer of Rheumatic Subjects before, during and after Attacks of the Disease*

Patient		Good health	Acute attack	Recovery	Recrudescence	Patient		Good health	Acute attack	Recovery	Recrudescence
		cc.	cc.	cc.	cc.			cc.	cc.	cc.	cc.
.....	N.D. =		0.005	0.03	0.003	Gi.....	N.D. =		0.002	0.02	
.....	" =	0.02	0.006		0.008	F.....	" =		0.02	0.02	
.....	" =	0.03	0.006	0.02		Ker.....	" =		0.002	0.002	0.0003
.....	" =		0.003	0.005	0.004	H.....	" =	0.02	0.005		
.....	" =		0.004	0.02		W.....	" =	0.03	0.004		
.....	" =		0.008	0.02	0.004						

Table XIV shows that in most patients the titer of antistreptolysin came high during the attack of acute rheumatism, returned to a normal level in quiescence and rose again during recrudescence. Patient S. had chronic suppurative otitis during the period of apparent

recovery; his titer changed only slightly. Patient Ker. had three severe rheumatic attacks within 8 months, and during the 3rd the titer was found to be extremely high. Patient F. with rheumatic heart disease developed pericarditis. Hemolytic streptococcus was not recovered from the throat; precipitins to the protein fraction of this organism were not detected in the serum. This was the only normal antistreptolysin titer obtained in this series of patients with frank carditis.

TABLE XV

*Antistreptolysin Titer during the Three Phases of the Rheumatic Attack*

Patient	Date	Clinical condition	Titer
R.	Apr. 13	Hemolytic streptococcus pharyngitis	cc. N.D. = 0.003
	" 18	Quiescent interval	" = 0.003
	May 5	Carditis and nephritis	" = 0.002
A.	Jan. 9	Hemolytic streptococcus pharyngitis	" = 0.04
	Feb. 6	End of quiescent interval	" = 0.02
	" 11	Fever, arthritis, carditis	" = 0.005
H.	Dec. 10	Rheumatism inactive	" = 0.02
	Mar. 12	Hemolytic streptococcus pharyngitis	" = 0.02
	" 17	Quiescent interval	" = 0.005
	" 31	Onset of attack	" = 0.004
	Apr. 5	Severe polyarthritis and carditis	" = 0.004
W.	Oct. 1	Rheumatism inactive	" = 0.02
	June 1	" "	" = 0.03
	Mar. 25	Hemolytic streptococcus pharyngitis	" = 0.04
	" 31	Quiescent interval	" = 0.03
	Apr. 5	" "	" = 0.03
	" 8	Fever, malaise, leucocytosis	" = 0.008
	" 13	Erythema marginatum	" = 0.004
	May 5	Polyarthritis	" = 0.004

Another study was made to determine the exact time relationship between the rise in antistreptolysin and the appearance of rheumatic manifestations. The findings are presented in Table XV.

Table XV illustrates first, the findings in rheumatic patients with a high titer and second, the findings in patients with a normal titer of antistreptolysin at the onset of hemolytic streptococcus infection. In the former, Patient R., there was little change in titer. The slight rise occurred on the day that carditis manifested itself (sudden development of prolonged conduction time P-R interval equal to 0.22

TABLE XIII

*Antistreptolysin Titer of Apparently Quiescent Rheumatic Subjects from Spring, 1928, to Fall, 1931*

Patient				Patient			
			cc.				cc.
Mul....	Jan. 14, 1932	Clinically inactive	N.D. = 0.02	Tre....	Jan. 7, 1931	Clinically inactive	N.D. = 0.03
Gill....	" 14, 1932	" "	" = 0.02	Wa....	Dec. 18, 1930	" "	" = 0.02
J. Jo....	" 14, 1932	" "	" = 0.007	Mo....	Feb. 4, 1932	" "	" = 0.02
J. Jo....	Oct. 8, 1930	" "	" = 0.01	Zi....	Nov. 1, 1930	" "	" = 0.05
Co....	June 10, 1931	" "	" = 0.01	Ro....	Jan. 23, 1932	" "	" = 0.03
Cou....	Jan. 14, 1931	" "	" = 0.03	Mah....	" 2, 1932	" "	" = 0.02
Ho....	Dec. 11, 1931	" "	" = 0.02	Bar....	" 7, 1931	" "	" = 0.03
Rom....	Jan. 7, 1932	" "	" = 0.02	La....	Oct. 4, 1930	" "	" = 0.006
Fl....	" 9, 1932	" "	" = 0.02	Mak....	" 4, 1930	" "	" = 0.009
Wy....	Oct. 1, 1930	" "	" = 0.02	Bus....	" 4, 1930	" "	" = 0.02
Sc....	June 10, 1931	" "	" = 0.02	Cer....	Jan. 7, 1931	" "	" = 0.02
Ba....	Oct. 1, 1930	" "	" = 0.03	Tri....	Dec. 17, 1930	" "	" = 0.04
D. Do..	Jan. 16, 1932	" "	" = 0.02	Len....	Jan. 28, 1932	" "	" = 0.03
Tr....	Nov. 1, 1930	" "	" = 0.009	Bea....	" 28, 1932	" "	" = 0.03
R. Ha..	Jan. 7, 1931	" "	" = 0.03	Hu....	" 29, 1932	" "	" = 0.009
Tol....	Dec. 13, 1930	" "	" = 0.005	Fe....	" 28, 1932	" "	" = 0.02

vals during health and disease. The findings are presented in Table XIV.

TABLE XIV

*Antistreptolysin Titer of Rheumatic Subjects before, during and after Attacks of the Disease*

Patient		Good health	Acute attack	Recovery	Recrudescence	Patient		Good health	Acute attack	Recovery	Recrudescence
		cc.	cc.	cc.	cc.			cc.	cc.	cc.	cc.
R.....	N.D. =		0.005	0.03	0.003	Gi.....	N.D. =		0.002	0.02	
G.....	" =	0.02	0.006		0.008	F.....	" =		0.02	0.02	
C.....	" =	0.03	0.006	0.02		Ker.....	" =		0.002	0.002	0.0008
S.....	" =		0.003	0.005	0.004	H.....	" =	0.02	0.005		
P.....	" =		0.004	0.02		W.....	" =	0.03	0.004		
O.....	" =		0.008	0.02	0.004						

Table XIV shows that in most patients the titer of antistreptolysin became high during the attack of acute rheumatism, returned to a normal level in quiescence and rose again during recrudescence. Patient S. had chronic suppurative otitis during the period of apparent

TABLE XVI

*A Comparison of Precipitin Formation with the Development of Antistreptolysin*

Patient	Time	Clinical condition	Antistreptolysin titer	*Precipitin reaction fraction	
				C17D	C17K
A. Non-rheumatic subjects					
Ho.	1st wk.	Hemolytic streptococcus pharyngitis	cc. N.D. = 0.01	—	—
	2nd "	" " pansinusitis	" = 0.0008	—	—
	3rd "	Subsiding sinusitis	" = 0.001	—	—
	4th "	Convalescence	" = 0.003	—	—
	5th "	Recovery	" = 0.006	—	—
O'H.	2nd "	Hemolytic streptococcus pneumonia	" = 0.004	—	—
	3rd "	Convalescence	" = 0.003	—	—
	4th "	"	" = 0.003	—	—
	6th "	Recovery	" = 0.003	—	—
B. Rheumatic subjects					
Maz.	2nd wk.	Acute rheumatic fever	cc. N.D. = 0.003	+	+
	3rd "	" " "	" = 0.003	+	+
	4th "	" " "	" = 0.003	+ ±	+ ±
	5th "	" " "	" = 0.003	++	++
	6th "	Subsiding " "	" = 0.003	+	+
	7th "	" " "	" = 0.004	±	±
	8th "	" " "	" = 0.004	±	±
	9th "	" " "	" = 0.004	±	—
	12th "	" " "	" = 0.004	—	—
	Wil.	1st "	Acute " "	" = 0.007	—
2nd "		" " "	" = 0.007	±	+
3rd "		" " "	" = 0.006	±	+
4th "		" " "	" = 0.007	+	+
5th "		Subsiding " "	" = 0.005		
†6th "		Hemolytic streptococcus pharyngitis	" = 0.02	Serum cloudy	
8th "		Rheumatic recrudescence	" = 0.007		
Man.	1st "	Acute rheumatic fever	" = 0.002	±	+
	2nd "	" " "	" = 0.002	+	+
	3rd "	Death	" = 0.002	+ ±	+ ±

\* Precipitin determinations represent overnight readings, uncentrifuged.

† The sudden fall in the titer of antistreptolysin has been observed in other patients at the onset of acute hemolytic streptococcus infection.

sin titer remained normal during the infection and the quiescent interval, but rose at the onset of the rheumatic attack. It reached a plateau level during the 1st week of rheumatism and did not increase after this. On the other hand, precipitins which were not detected

seconds). In the latter, Patients A. and H., the titer rose sharply on the day of onset of the rheumatic attack. In Patient W., under close observation, the quiescent interval ended on April 6. On April 8, vague symptoms and fever with leucocytosis were noted. The titer rose from an N.D. of 0.03 cc. on April 5 to an N.D. of 0.008 cc. on April 8. During the following days the titer of antistreptolysin reached a plateau level of an N.D. of 0.004 cc., and the patient developed skin and joint manifestations of active rheumatism.<sup>6</sup>

Finally, a study was made to compare the relationship of the development of antistreptolysin with the appearance of the precipitins in the rheumatic subject and in the non-rheumatic individual. For this purpose the sera of patients recovering from hemolytic streptococcus infection were tested at frequent intervals. The findings are presented in Table XVI.

From Table XVI it is seen that in the sera of two non-rheumatic subjects, Ho. and O'H., with hemolytic streptococcus infection, precipitins were not detected. Each of these individuals developed antistreptolysin. This titer remained at a high level for a period of weeks. The findings in these two individuals are representative. In the majority of non-rheumatic subjects recovering from hemolytic streptococcus infection, although precipitins were either weak or absent, antistreptolysin appeared in titer which remained at a constantly high level until after recovery. This was shown in the small group of patients convalescing from scarlet fever. Precipitin reactions were strong in only the one individual, who developed rheumatic fever; the antistreptolysin titer was high, however, in each instance.

In contrast, in most patients with acute rheumatism, both of these antibodies were detected. In nearly every instance the antistreptoly-

<sup>6</sup>Two exceptions have been noted. Patient Au., a rheumatic subject, contracted hemolytic streptococcus pharyngitis but failed to develop any clinical evidence of rheumatic fever. The titer of antistreptolysin remained N.D. = 0.02 cc. for 1 month. Patient Le. on the 7th day after hemolytic streptococcus pharyngitis developed acute rheumatism and infectious mononucleosis. The antistreptolysin titer remained constant, N.D. = 0.02 cc. throughout the illness. Dr. John Paul of New Haven studied samples of this patient's serum and detected the development of an heterophile antibody. Agglutinins to sheep red blood cells appeared in titer 1:128 during the 2nd week of the illness.

ject during the first two phases, the period of infection and the quiescent interval, and rises precipitously just before the appearance of rheumatic manifestations. Unlike the development of precipitins, the antistreptolysin titer reaches its high level during the 1st week of the rheumatic attack and maintains this constantly, irrespective of the patient's condition. Finally, the presence of antistreptolysin in titer of such a degree that 0.005 cc. of the patient's serum neutralizes  $2\frac{1}{2}$  minimal hemolytic doses of yeast extract streptolysin, is considered a specific indication of infection with hemolytic streptococcus. The constant finding of this high titer of antistreptolysin in the serum of patients with acute rheumatic fever is strong evidence that the rheumatic attack has been initiated by *Streptococcus hemolyticus*. There is a close relationship between the time of appearance of this antibody in the circulation and the manifestations of activity of the rheumatic process.

#### SUMMARY

In the first two papers findings were presented which point to a close relationship between the incidence of rheumatic fever and the distribution of *Streptococcus hemolyticus*. The fact was emphasized that in the rheumatic subject a recrudescence of the disease process is usually preceded by pharyngeal infection with hemolytic streptococci. These organisms conspicuous in the throat flora during the period of infection preliminary to an attack of acute rheumatism fell into six antigenic groups and produced toxins which in 70 per cent were neutralized by a monovalent streptococcus antiserum. In the present study, four series of observations have been presented, demonstrating the development of immune bodies to hemolytic streptococcus during the course of rheumatic fever. The agglutination and complement fixation reactions of sera from patients with acute rheumatism suggest recent infection with streptococcus. Precipitin tests indicate that at the time of appearance of the rheumatic attack, individuals develop, in their blood, precipitins to the protein fractions of hemolytic streptococcus. That these precipitins may not be entirely specific is recognized from their cross-reactions with antigens of chemically related organisms. The studies made in association with E. W. Todd of England have demonstrated that at the onset of an attack of acute



during the period of infection nor in the quiescent interval, appeared in weak concentration during the first few days of rheumatism. As the attack increased in severity, the precipitin reaction became more marked. With a certain degree of fluctuation, there was a parallelism between increasing intensity of the disease and the development of strong precipitins. Patient Maz. during the 3rd and 4th weeks appeared extremely ill, and precipitins were most marked in this period, but the antistreptolysin titer did not rise. In the case of Patient Man. with fulminating rheumatism, the antistreptolysin titer remained at a constant level and the precipitins increased in strength until the day of death. In those individuals who recovered, demonstrable precipitins disappeared rapidly, but the titer of antistreptolysin persisted at a high level for a long period of time.

#### *Summary of Antistreptolysin Determinations*

In the normal individual in good health, the titer of antistreptolysin is of such a degree that 0.01 cc. of serum neutralizes  $2\frac{1}{2}$  minimal hemolytic doses of yeast extract streptolysin. The antistreptolysin titer of patients convalescing from diseases other than hemolytic streptococcus infection is approximately normal. The antistreptolysin titer of patients during the acute stage of illness with hemolytic streptococcus infection is also approximately normal. In patients convalescing from infection with hemolytic streptococcus, the N.D. is approximately 0.005 cc. in most instances. The antistreptolysin titer of rheumatic subjects with inactive disease is usually normal or slightly elevated. The antistreptolysin titer of inactive rheumatic subjects tested after non-specific stimulation with an heterologous antigen either remains normal or falls to normal. In each rheumatic subject during an attack, following hemolytic streptococcus pharyngitis, the N.D. is approximately 0.005 cc. Likewise in each patient with acute rheumatism from whose throat hemolytic streptococcus was not recovered at the onset of the attack, the N.D. is also approximately 0.005 cc. Furthermore, in patients with acute rheumatism denying previous respiratory infection, the N.D. is also approximately 0.005 cc. The antistreptolysin titer falls slowly to normal during recovery and rises in each instance of recrudescence to an N.D. of 0.005 cc. The antistreptolysin titer remains normal in the rheumatic sub-

ject during the first two phases, the period of infection and the quiescent interval, and rises precipitously just before the appearance of rheumatic manifestations. Unlike the development of precipitins, the antistreptolysin titer reaches its high level during the 1st week of the rheumatic attack and maintains this constantly, irrespective of the patient's condition. Finally, the presence of antistreptolysin in titer of such a degree that 0.005 cc. of the patient's serum neutralizes  $2\frac{1}{2}$  minimal hemolytic doses of yeast extract streptolysin, is considered a specific indication of infection with hemolytic streptococcus. The constant finding of this high titer of antistreptolysin in the serum of patients with acute rheumatic fever is strong evidence that the rheumatic attack has been initiated by *Streptococcus hemolyticus*. There is a close relationship between the time of appearance of this antibody in the circulation and the manifestations of activity of the rheumatic process.

#### SUMMARY

In the first two papers findings were presented which point to a close relationship between the incidence of rheumatic fever and the distribution of *Streptococcus hemolyticus*. The fact was emphasized that in the rheumatic subject a recrudescence of the disease process is usually preceded by pharyngeal infection with hemolytic streptococci. These organisms conspicuous in the throat flora during the period of infection preliminary to an attack of acute rheumatism fell into six antigenic groups and produced toxins which in 70 per cent were neutralized by a monovalent streptococcus antiserum. In the present study, four series of observations have been presented, demonstrating the development of immune bodies to hemolytic streptococcus during the course of rheumatic fever. The agglutination and complement fixation reactions of sera from patients with acute rheumatism suggest recent infection with streptococcus. Precipitin tests indicate that at the time of appearance of the rheumatic attack, individuals develop, in their blood, precipitins to the protein fractions of hemolytic streptococcus. That these precipitins may not be entirely specific is recognized from their cross-reactions with antigens of chemically related organisms. The studies made in association with E. W. Todd of England have demonstrated that at the onset of an attack of acute

TABLE I  
*Reactivating Property of Blood Sera*

Material and dose of 1st intravenous injection per kilo of body weight	Material and dose of 2nd intravenous injection per kilo of body weight	Interval between skin and 1st intravenous injection	Interval between 1st and 2nd intravenous injection	Results		
				Total number of rabbits tested	No. of negative rabbits	No. of positive rabbits
20 units No. 1741* + 0.9 cc. No. 251† + 0.1 cc. No. 230† cc. 0.85% NaCl solution	—	hrs. 24	—	3	3	0
cc. rabbit Serum I	100 units No. 1741* + 0.9 cc. No. 251† + 0.1 cc. No. 230†	23½	½	3	3	0
" " " I	1 cc. rabbit Serum I	23½	½	3	3	0
" mixture 1 cc. rabbit Serum I + 1 cc. horse serum	—	24	—	3	3	0
cc. rabbit Serum I	—	24	—	3	3	0
" " " I	1 cc. human serum	24	—	3	3	0
" " " I	2 " mixture of 1 cc. No. 251† + 1 cc. No. 230†	24	—	3	3	0
" " " I	100 units No. 1741* + 0.9 cc. No. 251† + 0.1 cc. No. 230†	23½	½	3	0	3
" " " I	80 units No. 1741* + 0.9 cc. No. 251† + 0.1 cc. No. 230†	23½	½	3	1	2
" " " I	50 units No. 1741* + 0.9 cc. No. 251† + 0.1 cc. No. 230†	23½	½	3	1	2
" " " I	25 units No. 1741* + 0.9 cc. No. 251† + 0.1 cc. No. 230†	23½	½	3	2	1
" " " I	10 units No. 1741* + 0.9 cc. No. 251† + 0.1 cc. No. 230†	23½	½	3	3	0
" rabbit Serum I diluted 1:10	90 units No. 1741* + 0.9 cc. No. 251† + 0.1 cc. No. 230†	23½	½	3	1	2
" rabbit Serum I diluted 1:50	" "	23½	½	3	2	1
" rabbit Serum I diluted 1:100	" "	23½	½	3	3	0
" rabbit Serum I	" "	½	23½	6	4	2
it No. 1746*	1 cc. rabbit Serum I	23½	½	3	3	0
rabbit Serum I	½ unit No. 1746*	23½	½	3	3	0

Filtrate.

horse serum.

coccus reacting factors. Rabbits injected with 0.85 per cent NaCl solution showed no reactions with the neutralized mixtures. Moreover, the rabbit serum by itself injected twice within the same interval of time between the injections, displayed no reacting potency. Inasmuch as it was previously observed (2) that antigen-antibody interaction of heterologous blood sera *in vivo* brought about formation of reacting factors, rabbits were each separately injected with rabbit and normal human serum, rabbit and normal horse serum and with rabbit and immune horse sera (Nos. 251 and 230), respectively. The injections of completely neutralized reacting factors into these rabbits elicited no reactions.

The treatment of rabbits with the rabbit serum did not raise their susceptibility to the toxic filtrates alone.

From the above experiments it was concluded that the preliminary intravenous injection of the rabbit serum induced reactivation of completely neutralized meningococcus reacting factors. The reactivating property of the serum was titrated in a twofold manner:

1. Prepared rabbits were injected intravenously with the same amount of the rabbit serum (*i.e.*, 1 cc. of undiluted rabbit serum, per kilo of body weight) and divided into a number of groups of three.  $\frac{1}{2}$  hour later rabbits of each group received a single intravenous injection of a mixture of a varying number of toxic units mixed with a constant amount of immune serum. In these experiments untreated rabbits showed no reactions with 120 neutralized reacting units, whilst the treated ones gave reactions with 25 and no reactions with 10 neutralized reacting units. Thus, the smallest amount of neutralized reacting factors showing no reactions in treated rabbits was approximately 17 units. There was obtained, therefore, reactivation of six-sevenths of the neutralized reacting factors.
2. Rabbits were also treated intravenously with various amounts of the rabbit serum and tested with a constant amount of neutralized reacting factors. In this manner, a dose as small as 1 cc. of dilution 1:50 of the serum was shown to possess the reactivating property.

In one experiment the reactivating effect of the serum persisted for 23 $\frac{1}{2}$  hours. Longer periods of time were not studied.

#### *Reactivation of Completely Neutralized Meningococcus Reacting Factors in Vitro*

In the following experiments it was attempted to determine whether the rabbit serum added to the neutralized factors *in vitro* would display the above described reactivating property.

100 meningococcus reacting units completely neutralized as above (page 679) were mixed with rabbit Serum I undiluted and diluted 1:10 and 1:50, respectively. Each mixture was tested in a group of three rabbits. The rabbit serum undiluted and diluted 1:10 reactivated the neutralized mixture, inasmuch as all the rabbits tested showed reactions. The dilution 1:50 had no effect, since no reactions were obtained in the group.

### *Reactivating Property of Sera of Various Animal Species*

It was further of interest to determine the frequency with which the reactivating property occurs in sera of various animal species. The sera were tested either by the *in vitro* or the *in vivo* methods described above. The results are summarized in Table II.

TABLE II  
*Testing of Various Sera for Reactivating Property*

Animal species	Tests <i>in vivo</i>				Tests <i>in vitro</i>			
	Serum dilution	Total No. of sera	No. of positive sera	No. of negative sera	Serum dilution	Total No. of sera tested	No. of positive sera	No. of negative sera
Man.....	Undiluted	2	1	1	1:3	45	24	21
Horse.....	"	1	1	0	1:3	2	1	1
Rabbit.....	"	30	21	9	1:10	9	7	2
Guinea pig.....	"	4	3	1	1:5	1	1	0

As is seen from Table II, the reactivating property described was found in sera of man, horse, rabbit and guinea pig. About 72 per cent of rabbit and 53 per cent of human sera showed this property. The number of horse and guinea pig sera tested was too small to allow any statistical conclusions.

### *Effect of Reactivating Sera upon Completely Neutralized B. coli Reacting Factors*

The work reported thus far has dealt with the reactivation of neutralized reacting factors of the meningococcus. The experiments summarized in Table III were done in order to determine whether the property described was also effective against neutralized reacting factors of other bacterial species.

As seen from Table III, the reactivating property appears to be non-

specific, inasmuch as the same serum was capable of reactivating neutralized reacting factors of unrelated microorganisms (*i.e.*, meningococcus and *B. coli*). Further experiments in order to ascertain this point are under progress.

TABLE III  
*Reactivation of Neutralized B. coli Toxic Filtrates*

Skin-preparatory injection	Ingredients of mixture injected intravenously 24 hrs. after skin injection			Total dose per kilo of body weight	Results*
	Toxic filtrate	Immune serum	Reactivating serum		
0.25 cc. <i>B. coli</i> Filtrate 1768	20 units <i>B. coli</i> Filtrate 1768	0.25 cc. diluted 1:3 anti- <i>coli</i> horse Serum 230	None	cc. 1.25	0/3
" "	" "	" "	Rabbit Serum 716 1 cc. diluted 1:3	2.25	2/1
" "	5 units <i>B. coli</i> Filtrate 1768	" "	" "	2.25	3/0
" "	10 units <i>B. coli</i> Filtrate 1768	0.25 cc. diluted 1:2 anti- <i>coli</i> horse Serum 230	" "	2.25	2/1
0.25 cc. meningococcus Filtrate 1746	75 units meningococcus Filtrate 1746	0.9 cc. antimeningococcus Serum 251 + 0.1 cc. anti- <i>coli</i> Serum 230	" "	2	3/1

\* The numerator indicates the number of positive rabbits. The denominator indicates the number of negative rabbits. The sum of both is the total number of rabbits used.

#### *Deterioration of Reactivating Sera*

It has been noticed in the course of the work that the reactivating property may disappear on storage.

Thus, one guinea pig serum obtained on Mar. 25, 1932, showed a high reactivating potency in dilution 1:5 by the *in vitro* method. On Mar. 31, 1932, the same serum was inactive in dilution 1:5. Lower dilutions were not tested. Also, rabbit Serum 634 possessed reactivating property in dilution 1:15 on Mar. 28, 1932. Dilution 1:10 of the same serum had no reactivating potency on Apr. 4, 1932.

The observations suggested studies on the relation of the reactivating property to the complement, and also on their heat resistance.

### *The Relation between Reactivating Property and Complement*

Rabbit Sera 654, 634 and 749 and guinea pig Sera 1, 2, 3 and 4 were employed. All the sera were obtained within 20 hours prior to the tests and kept in the refrigerator until used.

The reactivating property was determined by *in vitro* addition (page 679) of undiluted sera to 100 neutralized meningococcus reacting units. All the sera tested were found to contain reactivating factors.

The complement titrations of the same sera were done simultaneously with the above tests. Amounts of each serum tested were 0.01, 0.05, 0.01 and 0.2 cc. These amounts were each mixed with 2 units of sheep red blood cell amboceptor (*i.e.* 0.1 cc. of 1:50 dilution) and 0.5 cc. of 5 per cent suspension of sheep red blood cells. The mixtures were incubated in a water bath at 37°C. for 30 minutes. All the guinea pig sera contained active complement, whilst the rabbit sera had no complement.

The experiments demonstrated that there was no apparent relationship between complement and the reactivating property of sera.

### *Heat Resistance of Reactivating Factors*

The results of the experiments are summarized in Table IV.

As seen from Table IV, the reactivating factors are heat-labile, the exposure to 56°C. for 5 minutes, 50°C. for 25 minutes and 37°C. for 4 hours being sufficient to destroy them.

Taking advantage of the above observations it was planned to determine whether immune sera contained native reactivating property, and if so, whether heating to a temperature destructive to the reactivating property but ineffective against neutralizing antibodies would raise the neutralizing potency of these sera.

### *Increase in Neutralizing Titer after Destruction of Reactivating Property*

Various immune sera, unheated and heated in the water bath at 37°C. for 4 hours were titrated for neutralizing antibodies in the usual manner (3). The antimeningococcus sera were tested with and without the auxiliary antibody. The results are recorded in Table V.

As seen from Table V, heating of immune sera at 37°C. for 4 hours substantially raised their neutralizing titers. Thus, the heated anti-

TABLE IV  
*Heat Resistance of Reactivating Factors*

Undiluted serum*		Temperature	Time of exposure	Results
		°C.	hrs.	
Rabbit Serum 718.....		Water bath, 37°C.	2	—
" " 718.....		" " 37° "	4	+
Horse " 7/561.....		" " 37° "	4	+
Rabbit " 634.....		" " 50° "	min.	—
" " 714.....		" " 50° "	15	+
" " 634.....		" " 56° "	25	+
" " 525.....		" " 56° "	5	+
" " 563.....		" " 56° "	20	+
		" " 56° "	20	+

— = no effect upon the reactivating property.  
+ = reactivating property destroyed.

\* The sera were tested for reactivating property on the days of these experiments.

TABLE V  
*Increase of Neutralizing Titer of Sera after Destruction of Reactivating Property*

Serum	Temperature exposure prior to titration	Reactivating property	CN titer without auxiliary antibody	CN titer with auxiliary antibody
Antimeningococcus horse Serum 7/540	None	+	<30 units in 1 cc.	20 units in 1 cc.
" " " "	Water bath, 37°C. 4 hrs.	—	50 units in 1 cc.	Not tested
Antimeningococcus horse Serum 7/561	None	+	Not tested	20 units in 1 cc.
" " " "	Water bath, 37°C. 4 hrs.	—	80 units	Not tested
Antityphoid horse Serum 57/560	None	+	375 units in 0.25 cc.	" "
" " " "	Water bath, 37°C. 4 hrs.	—	600 units in 0.25 cc.	" "

CN = the largest number of reacting units completely neutralized by the amount of serum indicated.

typhoid horse serum showed a 60 per cent increase in the titer. Moreover, the treatment enabled the antimeningococcus horse Sera 7/540 and 7/561 to neutralize completely 50 and 80 units of meningococcus reacting factors, respectively. In contrast, only irregular neutraliza-



tion was obtained with the same unheated sera without the aid of the auxiliary antibody and only 20 units were completely neutralized even with the aid of the auxiliary antibody.

Incidentally, in the course of some work on antibody absorptions, Dr. H. Klein tested in this laboratory for control purposes the neutralizing potency of immune antityphoid serum diluted 1:10 after it was incubated for several hours in a water bath at 37°C. There was obtained by him a striking increase in the neutralizing titer.

As also seen from Table V, the immune sera contained native reactivating property. The heating at 37°C. which brought about the increase in the neutralizing potency was coincident with destruction of the reactivating property.

It appears, therefore, that the native reactivating property of immune sera interferes with their neutralizing potency.

#### DISCUSSION AND CONCLUSIONS

In a series of publications (1, 3) observations have been reported concerning serum neutralization of reacting factors of the phenomenon of local skin reactivity to bacterial filtrates. When various amounts of a toxic filtrate are titrated against a constant amount of immune serum it is possible to determine the largest amount of filtrate which is completely neutralized (*i.e.* in all rabbits tested) by this amount of serum and, thus, to express quantitatively the neutralizing potency of the serum (CN titer). If the titration is continued beyond the CN titer (*i.e.* in greater quantity of toxin) there is usually observed a rather protracted zone of irregular neutralization (IN titer) in which the percentage of positive rabbits is considerably lower than in the control group. If still larger amounts of toxic filtrate are titrated one arrives at a point when the addition of serum produces no effect. The CN titer of *B. typhosus* and *B. coli* toxins is well demarcated. There is consistently obtained complete neutralization of these filtrates in multiple proportions. The antimeningococcus sera behave differently. The CN titer of these sera is either nil or very low, whilst the IN titer may be high. Moreover, there is no proportionate increase in the neutralizing potency when multiples of the CN titer are used. *In vitro* addition of the auxiliary antibody (certain heterologous immune sera) to the antimeningococcus sera corrects this irregularity,

inasmuch as the mixture acquires the power of complete neutralization of meningococcus reacting factors in multiple proportions.

The effect of certain blood sera described in this paper consists in the restoration of toxicity of completely neutralized bacterial filtrates; *i.e.*, an effect diametrically opposed to the auxiliary antibody. The sera do not increase the toxicity (*i.e.* reacting potency) of the filtrates themselves. For these reasons, the property or factors can be termed reactivating.

The reactivating factors have no apparent relationship to the complement content. They are heat-labile, deteriorate on storage and can be accurately measured by the *in vitro* and *in vivo* methods described in the text. The native reactivating factors present in the immune sera can be destroyed at a temperature (*i.e.* at 37°C. for 4 hours) which has no destructive effect upon the neutralizing antibodies. The destruction of the factors is, then, coincidental with a considerable increase of the neutralizing potency of immune sera. The therapeutic effect of sera treated in this manner remains to be determined.

Moreover, the heated antimeningococcus immune sera are capable of complete neutralization of meningococcus reacting factors without the aid of the auxiliary antibody. This simplifies the determinations of the neutralizing potency and allows its expression in terms of the CN titer which is well demarcated.

The immunological significance of the reactivating factors warrants consideration, inasmuch as they appear to be non-specific, widely distributed among various species of animals (*i.e.* man, horse, rabbit and guinea pig) and are capable of displaying the effect *in vivo*. It is conceivable that they may play an important antagonistic rôle if present in therapeutic immune sera; *i.e.*, interfering both with the effect of passively acquired antibodies and also, possibly, restoring toxicity to toxic substances neutralized by actively acquired antibodies.

The possible reactivating effect of normal human blood sera (*i.e.* about 53 per cent of sera tested) should be guarded against in performing blood transfusions in cases of infectious diseases.

Studies on the interrelationships between the auxiliary antibody and the reactivating property and also on the effect of the latter upon various antibodies reported are under progress.

## SUMMARY

In this paper there are reported observations on a certain new reactivating property of normal and immune blood sera of various animal species. The effect of the reactivating sera consists in restoration of the toxicity *in vivo* and *in vitro* of completely neutralized meningococcus and *B. coli* reacting factors.

The property is apparently non-specific, heat-labile, lessens on storage, and has no relationship to complement.

Heating of immune sera to a temperature destructive for the reactivating property but innocuous to the neutralizing antibodies, raises considerably their neutralizing potency.

The possible immunological significance of the reactivating property is discussed in this paper.

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# PHENOMENON OF LOCAL SKIN REACTIVITY TO BACTERIAL FILTRATES: FORMATION OF REACTING FACTORS IN VIVO

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It has been reported in previous communications (1) that intravenous injections of mixtures of serum precipitinogens with precipitating antisera produced severe hemorrhagic necrosis in rabbit skin sites prepared with bacterial filtrates. The reacting potency of the mixtures did not depend on the degree of turbidity formed. Also, clear supernatant fluids obtained by centrifugalization of partially precipitated sera elicited severe reactions. It was concluded from these and other observations that the reacting potency of the mixtures was not due to the mechanical effect of colloidal particles in the blood stream but to some toxic factors liberated or formed in the sera through the colloidal disturbance of the antigen-antibody interaction. The purpose of the work embodied in the present paper was to determine whether the reaction between the antigen and antibody induced *in vivo* would bring about formation of reacting factors in the blood stream.

## *Formation of Reacting Factors in Actively Sensitized Rabbits*

In this part of the work rabbits were sensitized by single or repeated intravenous injections of animal proteins. After various intervals of time they were prepared by single intradermal injections of 0.25 cc. of undiluted bacterial "agar washings" filtrate. The skin-preparatory potency of the filtrates employed was ascertained in the usual manner prior to the experiments (2). 24 hours after the skin preparation the rabbits received single intravenous injections of the animal protein to which they were previously sensitized (*i.e.*, test injections). For repeated skin preparations in retests with the same or various proteins, a different bacterial filtrate was employed each time in order to avoid acquirement of specific active immunity (3). They were *B. typhosus*, meningococcus Group III (44B strain), meningococcus Group I (44 D strain) and *B. coli* "agar washings" filtrates, re-

TABLE I  
*Formation of Reacting Factors in Sensitized Rabbits*

Sensitizing antigen	Doses of sensitizing injections per kilo of body weight				Interval of time between sensitizing injections			Antigens and doses used for test injections per kilo of body weight				Incubation period <i>days</i>	Result of test injections*				
	1st	2nd	3rd	4th	1st <i>days</i>	2nd <i>days</i>	3rd <i>days</i>	4th <i>days</i>	1st	2nd	3rd		4th	1st	2nd	3rd	4th
Egg albumin	5% solution†	5% solution	5% solution	—	7	7	—	—	5% egg albumin†	Undiluted horse serum†	—	—	2	4/0	0/4	—	—
“	“	“	“	—	7	7	—	—	Undiluted horse serum†	5% egg albumin†	—	—	2	0/4	4/0	—	—
Horse serum	1 cc. undiluted	1.5 cc. undiluted	2 cc. undiluted	3 cc. undiluted	7	7	7	—	Undiluted rabbit serum†	Undiluted horse serum†	—	—	7	0/2	2/0	—	—
“	“	—	—	—	—	—	—	—	Undiluted horse serum†	—	—	—	12	6/0	—	—	—
“	Diluted 1:10	—	—	—	—	—	—	—	“	—	—	—	8	2/1	—	—	—
“	Diluted 1:100	—	—	—	—	—	—	—	“	—	—	—	8	0/2	—	—	—
“	Diluted 1:1,000	—	—	—	—	—	—	—	“	—	—	—	8	0/3	—	—	—
“	Diluted 1:10,000	—	—	—	—	—	—	—	“	—	—	—	8	0/3	—	—	—

Horse serum	Undiluted†	—	—	—	—	—	—	—	Horse serum diluted 1:10	Horse serum diluted 1:10	—	—	—	7	0/4/3/1	—	—
"	"	—	—	—	—	—	—	—	Horse serum diluted 1:10	Horse serum diluted 1:10	—	—	—	7	0/3/0/3	—	—
"	Diluted 1:100	—	—	—	—	—	—	—	Horse serum diluted 1:100	Horse serum diluted 1:100	—	—	—	7	0/2/2/0	—	—
"	"	Diluted 1:100	—	—	—	—	—	—	Horse serum diluted 1:10	Undiluted human serum†	Undiluted sheep serum†	Undiluted goat serum†	—	7	4/0/0/4/0/4/0/4	—	—
"	Undiluted†	—	—	—	—	—	—	—	Horse serum diluted 1:100	Undiluted horse serum†	—	—	—	2	0/4/0/4	—	—
"	"	"	—	—	—	—	—	—	Undiluted human serum†	Undiluted guinea pig serum†	—	—	—	8	0/3	—	—
"	"	"	—	—	—	—	—	—	Undiluted guinea pig serum†	Undiluted sheep serum†	—	—	—	8	0/3	—	—
"	"	"	—	—	—	—	—	—	Undiluted sheep serum†	—	—	—	—	8	0/3	—	—

\* The numerator indicates the number of positive rabbits. The denominator indicates the number of negative rabbits. The sum of both indicates the total number of rabbits used.

† 1 cc.

TABLE I—*Concluded*

Sensitizing antigen	Doses of sensitizing injections per kilo of body weight				Interval of time between sensitizing injections			Antigens and doses used for test injections per kilo of body weight				Incubation period days	Result of test injections*			
	1st	2nd	3rd	4th	1st 2nd days	2nd 3rd days	3rd 4th days	1st	2nd	3rd	4th		1st	2nd	3rd	4th
Horse serum	1 cc. undiluted	2 cc. undiluted	3 cc. undiluted	4 cc. undiluted	7	7	1	Undiluted sheep serum†	—	—	—	7	3/0	—	—	—
“ “	“ “	“ “	“ “	“ “	7	7	1	Undiluted guinea pig serum†	Undiluted horse serum†	—	—	7	0/3 3/0	—	—	—
Rabbit serum	Undiluted†	—	—	—	—	—	—	Undiluted rabbit serum†	—	—	—	8	0/3	—	—	—
Human serum	“	—	—	—	—	—	—	Undiluted human serum†	—	—	—	7	2/1	—	—	—
“ “	Diluted 1:10,000	Diluted 1:10,000†	—	—	1	—	—	Undiluted human serum†	Undiluted horse serum†	—	—	8	2/0 0/2	—	—	—
“ “	Diluted 1:100,000	Diluted 1:100,000†	Diluted 1:100,000†	—	1	1	—	Undiluted human serum† “ “	—	—	—	8	0/2	—	—	—

spectively. These experiments are summarized in Table I. In this table and others of this paper, the expression positive rabbit means that there was obtained in the rabbit's prepared skin site a severe hemorrhagic and necrotic lesion 4 to 5 hours after the test intravenous injection. The lesions which were intense in the majority of rabbits were characteristic of the phenomenon of local skin reactivity to bacterial filtrates (4). By negative rabbit is meant absence of skin reaction following the test intravenous injection.

As is seen from Table I, rabbits actively sensitized to animal proteins (*i.e.*, blood sera and egg albumin) responded with formation of reacting factors *in vivo* upon intravenous reinjection of the same protein after an adequate incubation period.<sup>1</sup>

The factors were apparently formed immediately after the test intravenous injection, inasmuch as the reactions in the prepared skin sites had developed to their fullest extent 4 to 5 hours later.

The state of active sensitization could be demonstrated in the manner described 7 to 8 days after a single intravenous injection and it persisted for longer periods of time (*i.e.*, 12 days). Shorter incubation periods were inadequate (*i.e.*, 2 days). The state could be also attained with diluted sera. A single injection of 1 cc. horse serum diluted 1:10 induced sensitization after 8 days. Higher dilutions of horse serum also elicited it but only upon repeated sensitizing injections and after longer incubation periods (*i.e.*, dilution 1:100, 14 days<sup>2</sup>). The human serum appeared to be of high antigenicity for rabbits because even 1 cc. of dilution 1:10,000 injected twice produced sensitization 8 days after the second sensitizing injection. The antigen for test injections could also be used in comparatively small amounts (*i.e.*, 1 cc. horse serum diluted 1:100).

The experiments of Table I also demonstrated the specificity of the sensitization described. Rabbits sensitized to small amounts of horse serum, while showing reactions with the same serum, gave no reactions upon retests with human, guinea pig, rabbit, sheep and goat sera. Similarly, rabbits sensitized to human serum showed reactions

<sup>1</sup> The term sensitization employed in this paper is limited to the type described.

<sup>2</sup> The term incubation period in the text and tables refers to the interval of time between the last sensitizing and the first test injection. When repeated sensitizing injections were made the true incubation period which was necessary, remained unknown.



with human serum but failed to respond to horse serum; and those sensitized to egg albumin did not react with horse serum. However, the specificity displayed by serum proteins of various animal species did not hold when repeated sensitization with large doses was extended over prolonged periods of time. Thus, rabbits sensitized by four intravenous injections of undiluted horse serum in doses of 1 to 4 cc., per kilo of body weight, reacted with sheep serum after an incubation period of 22 days, although failing to respond to guinea pig serum. Sera of these rabbits obtained before the test injections were titrated for precipitins against sheep and guinea pig sera. They gave precipitation with sheep serum diluted 1:20 and no precipitation with guinea pig serum. It was shown, therefore, that the cross-reactions with heterologous sera were coincidental with cross-precipitations. In a recent paper, Hektoen and Cole offered an explanation for cross-precipitation obtained after employment of large doses of antigen (5).

#### *Formation of Reacting Factors in Passively Sensitized Rabbits*

The purpose of the work now to be taken up was to determine whether reacting factors would form *in vivo* in passively sensitized rabbits. Rabbits were prepared by a single intradermal injection of *B. typhosus* ( $T_L$ ) "agar washings" filtrate and 23½ and 24 hours later injected intravenously, first, with the antigen, and secondly, with the antibody, respectively. In some experiments the order of intravenous injections was reversed; in others the interval of time was varied. Parallel experiments were done in which mixtures of the antigen with the antibody made *in vitro* were injected intravenously into prepared rabbits. The results are summarized in Table II.

As seen from Table II, two separate intravenous injections of precipitinogen-containing serum (*i.e.*, normal human serum) and precipitating antiserum (*i.e.*, anti-human horse serum) elicited severe hemorrhagic necrosis in skin sites prepared with bacterial filtrates. No reactions were obtained when each of the sera was injected alone in the same dose; when either the anti-human horse serum or the normal human serum was reinjected twice, or when there were made two separate injections of normal horse and normal human sera. The experiments demonstrated, therefore, formation of reacting factors *in vivo* through the interaction of passively acquired antigen and antibody.

## Formation of Reacting Factors in Passively Sensitized Rabbits

Intravenous injections		Interval between skin and intravenous injections	Interval between 1st and 2nd intravenous injections	Results
1st	2nd			
2 cc. horse Serum 416	—	hrs. 24	—	0/3
1 " human " I	—	24	—	0/3
1 " horse " 416	1 cc. horse Serum 416	23½	½	0/6
1 " human " I	1 " human " I	23½	½	0/3
1 " " " SL	1 " horse " 416	23½	½	0/3
1 " " " 918	1 " " " 416	23½	½	0/3
1 " " " B	1 " " " 416	23½	½	0/3
1 " " " Sc	1 " " " 416	23½	½	0/3
1 " " " 416	1 " human " XIII	23½	½	0/3
1 " " " 416	1 " " " XIV	23½	½	0/3
1 " anti-human horse Serum 7/405	1 " " " I	23½	½	0/3
Mixture of 1 part human serum—1 part anti-human horse Serum 7/405	—	24	—	3/0
1 cc. human Serum I	1 cc. anti-human horse Serum 7/405	23½	½	3/0
1 " " " I diluted 1:10	" "	23½	½	3/0
1 cc. human Serum I	1 cc. anti-human horse Serum 7/486	23½	½	3/0
1 " " " I	1 cc. anti-human horse Serum 7/501	23½	½	0/3
1 " " " I	1 cc. anti-human horse Serum 7/515	23½	½	0/3
1 " " " I	1 cc. anti-human horse Serum 7/522	23½	½	2/1
1 " " " I	1 cc. anti-human horse Serum 7/527	23½	½	0/3
1 " " " I	1 cc. anti-human horse Serum 7/540	23½	½	0/3
1 " " " I	1 cc. anti-human horse Serum 7/486	min. 15	23½	1/2
Human serum 2 cc. Group III	1 cc. 5% human red blood cells Group II	hrs. 23½	½	2/6
Pneumococcus Type III culture filtrate 3 cc.	—	24	—	0/4
1 cc. Antipneumococcus Type III horse serum	—	24	—	0/3
3 cc. Pneumococcus Type III culture filtrate	1 cc. Antipneumococcus Type III horse serum	23½	½	5/1
1 cc. human Serum I	1 cc. anti-human horse Serum 7/486	24 hrs. prior to skin injection	24	0/3

Positive rabbits were obtained when the interval of time between the injections of serum precipitinogen and precipitating antiserum was  $\frac{1}{2}$  hour, and also, in one experiment, when it was  $23\frac{3}{4}$  hours. A 48 hour interval gave negative results.

In the majority of experiments of Table II antigen was injected first and the antibody second. No reactions were elicited when the order of intravenous injections was reversed. Precipitation titrations with the serum precipitinogen and precipitating antiserum showed that there were required larger amounts of antibody than antigen for the precipitation. Thus, human serum diluted 1:1,000 precipitated with undiluted

TABLE III  
*Precipitation Titer of Anti-Human Horse Sera*

Undiluted precipitating antisera	Precipitino-gen sera	Precipitation results with various dilutions of precipitinogen				Formation of reacting factors <i>in vivo</i>
		1:2	1:10	1:100	1:1,000	
Human sera	7/405	3+	3+	4+	±	※
" "	7/486	4+	4+	2+	—	※
" "	7/501	4+	4+	4+	±	0
" "	7/515	2+	2+	2+	—	0
" "	7/522	4+	4+	1+	—	※
" "	7/527	4+	4+	1+	—	0
" "	7/540	4+	4+	2+	—	0

※ = serum induced formation of reacting factors *in vivo*.

0 = serum did not induce formation of reacting factors *in vivo*.

anti-human horse serum, whilst the antibody diluted 1:40 already failed to precipitate with undiluted and diluted 1:10 human serum. Inasmuch as unquestionably a part of the injected serum rapidly disappears from the blood stream, it is conceivable that in the latter experiment the reactions did not take place because an insufficient amount of antibody remained in the blood stream at the time of the subsequent injection of the antigen.

As is also seen from Table II, there were tested various bleedings of Horse 7. Since every bleeding did not give positive results it seemed of interest to determine their precipitation titers (Table III).

As is seen from a comparison of the respective results of Tables II and

III, no relationship could be established between the precipitin contents and the ability of the sera to induce formation of reacting factors upon combination with the precipitinogen *in vivo*.

The results of Table II demonstrate in addition that the interaction of inactive *Pneumococcus* Type III culture filtrates with Antipneumococcus Type III horse serum induced formation of reacting factors *in vivo*. This observation is corroborated by previously reported results on the reacting potency of mixtures of the same filtrates and sera made *in vitro* (6).

There was obtained suggestive evidence that the factors could also be formed through the interaction of hemagglutinins with human red blood cells. The results are not sufficiently conclusive to warrant special consideration in this paper. Further work is in progress.

No clear-cut results could be obtained as yet concerning formation of reacting factors *in vivo* through the interaction either of homologous (*i.e.* rabbit) antiserum with heterologous serum or heterologous antiserum with homologous (*i.e.* rabbit) serum. Further experiments are also under way.

#### *Effect of Reacting Factors Formed in Vivo upon Skin Sites Prepared by Non-Bacterial Substances*

In order to emphasize the essential rôle of bacterial filtrates in the phenomenon under discussion the experiments embodied in Table IV were done.

As seen from Table IV, no reactions were obtained when rabbits were injected intradermally, either with the serum precipitinogen or the precipitating antiserum, and after various intervals of time reinjected intravenously with the precipitating antiserum in the case of the former, and with the serum precipitinogen in the case of the latter. Moreover, whilst mixtures of serum precipitinogen with precipitating antiserum injected intravenously elicited severe hemorrhagic necrosis in skin sites prepared with a potent bacterial filtrate, they had no effect upon sites prepared either with the precipitinogen or the precipitins. Also, an intravenous injection of horse serum into rabbits actively sensitized to the serum produced no effect either upon areas in which there was induced inflammation with turpentine, or in areas prepared with horse serum. As is seen from experiments

TABLE IV

*Effect of Reacting Factors Formed in Vivo upon Sites Prepared with Non-Bacterial Substances*

Preliminary treatment	Skin-preparatory injection*	Test intravenous injection per kilo of body weight	Interval between skin and intravenous injection	Results†
			hrs.	
None	Human serum	1 cc. anti-human horse Serum 4/405	1	0/3
"	" "	" "	4	0/3
"	" "	" "	6	0/3
"	" "	" "	24	0/3
"	" "	2 cc. of mixture of 1 part of 4/405—1 part human serum diluted 1:4	24	0/3
"	Anti-human horse Serum 7/405	" "	24	0/3
"	<i>B. typhosus</i> culture filtrate	" "	24	3/0
"	Anti-human horse Serum 7/405	1 cc. human serum	24	0/3
1 cc. horse serum intravenously 1 wk. prior to skin injection	<i>B. typhosus</i> culture filtrate	1 " horse "	24	3/0
" "	Turpentine diluted 1:5	1 " " "	24	†0/4
" "	Horse serum	1 " " "	24	0/6
" "	" "	25 <i>B. typhosus</i> reacting units	24	0/3

\* 0.25 cc. of undiluted material was used for each intradermal injection.

† The numerator indicates the number of positive rabbits, the denominator the number of negative rabbits. The sum indicates the total number of rabbits tested.

‡ Abscesses, no hemorrhagic necrosis.

of Tables I and IV, an intravenous injection of horse serum into sensitized rabbits invariably produced severe lesions at the sites prepared with a bacterial filtrate.

Thus, the experiments clearly showed that the antigen-antibody interaction elicited in the manner described, while producing no effect

upon normal, sensitized and inflamed tissues, was responsible for severe injury to tissues prepared with bacterial filtrates.

*Effect of Intradermal Reinjection of Antigen-Antibody Mixture upon Sites Prepared with Bacterial Filtrates*

There remained the question whether the antigen-antibody interaction taking place outside the blood stream would also bring about a similar injurious effect upon skin sites prepared with bacterial filtrates. The following experiments were done.

Six rabbits each received a single intradermal injection of 0.25 cc. of undiluted *B. typhosus* ( $T_L$ ) "agar washings" filtrate. 24 hours later the same areas were each reinjected with 0.5 cc. of a mixture consisting of one part of anti-human horse Serum 7/527 and one part of human serum diluted 1:4. The same mixture was also injected intravenously into three rabbits prepared by a single intradermal injection of the above *B. typhosus* filtrate 24 hours before. The rabbits reinjected intradermally showed no hemorrhagic necrosis during the following 48 hours of observation. The rabbits reinjected intravenously had severe hemorrhagic and necrotic lesions at the prepared skin sites 4 to 5 hours later.

These experiments demonstrate that the *sine qua non* of the phenomenon is that the antigen-antibody mixture be brought into contact with the prepared skin areas *via* the blood stream.

DISCUSSION AND CONCLUSIONS

The data presented in this paper offer a new method for the demonstration of antigen-antibody interaction. It is easily carried out, as follows:

A rabbit sensitized to some animal protein receives an intradermal injection of 0.25 cc. of undiluted bacterial filtrate of ascertained skin-preparatory potency. 24 hours after the skin preparation the rabbit is injected intravenously with the same animal protein. From 4 to 5 hours later there appears severe hemorrhagic necrosis at the prepared skin site.

Inasmuch as the necessary state of sensitization can be obtained by a single intravenous injection of a protein and a 1 week incubation period is sufficient, the method offers the advantages of speed and simplicity. The readings are reliable and clear-cut since the incidence of positive rabbits is high (*i.e.* with some proteins about 85 per cent

of animals tested after a single sensitizing injection) and the severe hemorrhagic necrosis makes the reaction unmistakable. The test is highly sensitive and strictly specific, unless there are made repeated sensitizing injections of large doses of the antigen. Anaphylactic shock in rabbits is difficult to elicit, and the test injections of the proteins do not have lethal effect upon these animals.

It is also possible to elicit severe reaction in the prepared skin site of non-sensitized rabbits upon separate intravenous injections of antigen and antibody (*i.e.*, passive transfer).

The test described is clearly differentiated from the Arthus phenomenon by the following important features.

(1) A single sensitizing injection is sufficient. (2) The reaction can be obtained in rabbits which are not ready as yet for the Arthus phenomenon. (3) Skin preparation with a bacterial filtrate of ascertained skin-preparatory potency is essential. (4) The reaction is limited to the prepared skin site. (5) The antigen-antibody interaction has to take place in the blood stream.

It has been previously shown (1) that similar reactions can be obtained in prepared rabbits by an intravenous injection of antigen-antibody mixture made *in vitro*. Considerable evidence has been accumulated to prove that the injury was not due to the mechanical effect of the precipitate in the blood stream but to a toxic principle formed through the antigen-antibody interaction. In this paper there is presented additional proof of the fact, inasmuch as the precipitation titer bears no relation to the resulting toxic effect of separately injected serum precipitinogens and precipitating antisera.

The part of the phenomenon under consideration in this paper deals with toxic factors formed *in vivo* through antigen-antibody interaction. These factors are capable of inducing *via* the blood stream severe injury in tissues prepared by potent bacterial filtrates. As was pointed out in previous papers (7) and also shown again in this paper, the preparatory injection of a bacterial filtrate brings about a transient state of vulnerability in the animal cell.<sup>3</sup> Vulnerability may or may not be associated with active inflammation. A great number of observations have afforded unquestionable proof that the primary

<sup>3</sup> The generalization to the animal cell is justified because the state of vulnerability was demonstrated in animals of various species and in various organs and tissues (11).

injection may induce vulnerability in spite of complete absence of primary erythema and may fail to elicit it in the face of active inflammation produced either by certain inactive bacterial filtrates or by non-bacterial inflammatory substances (*i.e.* broth, turpentine, gum tragacanth, egg albumin, horse serum, blood plasma, India ink, agar, gelatine, sodium arsenate, heparin, histamine, spirits of nitroglycerine, eserine hydrochloride, trypan blue, adrenaline, pituitrin, silicic acid, charcoal, infusorial earth, paraffin oil and caseine). Recently Opie (8), Menkin (9) and Cannon and Pacheco (10) emphasized fixation of colloidal particles in the blood stream by inflamed tissues. If one should grant that some sort of undetectable inflammation is invariably elicited by skin injection of a bacterial filtrate, then it is easily understood why the reacting factors formed or introduced into the blood stream localize in the prepared area. However, this alone does not explain why a severe reaction takes place only in areas prepared with bacterial filtrates of ascertained skin-preparatory potency and fails to occur in other inflamed areas. For these reasons, it must be concluded beyond any doubt that the bacterial filtrates described (*i.e.* those of high skin-preparatory potency) possess in addition the ability of inducing a certain state of vulnerability. Thus, the vulnerable cell becomes a reagent for the detection of apparently widely separated groups of toxic principles, all with one characteristic feature in common; *i.e.*, the production of injury *via* the blood stream. These principles are as follows:

1. *Neutralizable and Antigenic Exotoxins*.—In previous communications it has been demonstrated that bacterial substances identical or closely related to true exotoxins are capable of eliciting severe injury in the vulnerable cell. The observations lead to the demonstration of a new category of antigenic and neutralizable exotoxins with a distinct difference between them and true exotoxins in the mechanism of the effect. The classical toxins are capable of primary local injury, whilst those under discussion inflict injury *via* the blood stream on vulnerable tissues. It has been previously assumed (13) that these toxins act as agents which produce a disturbance in the colloidal state of the blood. As a result, certain toxic factors are formed *in vivo* which attack the vulnerable cell.

2. *Formation of Reacting Factors Resulting from Intravenous Injection of Agar*.—It has been shown by Sickles (12) and corroborated by



the present author (1) that agar, whilst possessing no skin-preparatory potency, when injected intravenously is capable of eliciting the reaction in areas prepared with potent bacterial filtrates. Inasmuch as the agar produces the reaction only *via* the blood stream, it may be concluded that it also acts as an agent producing a disturbance in the colloidal state of the blood, necessary for the formation of reacting factors injurious to the vulnerable cell. It has been demonstrated by Bordet (13) that addition of agar to blood serum induces formation of anaphylatoxins. It has been further observed by Novy and De Kruif (14) that agar also forms anaphylatoxins *in vivo*. Thus, correlative studies on anaphylatoxins and the phenomenon under discussion suggest themselves.

3. *Formation of Reacting Factors Resulting from in Vivo Interaction of Non-Bacterial Antigens with Antibodies.*—As described in this paper, the antigen-antibody combination *in vivo* brings about formation of toxic factors which are detected by the vulnerable cell. It also remains to determine whether the toxic principles formed by the antigen-antibody combination of anaphylaxis and Arthus phenomenon are similar to the reacting factors of the phenomenon under discussion. It is conceivable that identical toxic factors may produce different effects in various tissues, the vulnerable cell of this phenomenon responding with hemorrhagic necrosis.

4. *Formation of Reacting Factors Resulting from in Vivo Interaction of Bacterial Antigens with Antibodies.*—It has been previously reported that mixtures of inactive pneumococcus filtrates with homologous antisera are capable of eliciting severe reactions in skin sites with induced vulnerability. In this paper it has been shown that separate ( $\frac{1}{2}$  hour apart) intravenous injections of the inactive filtrate and the serum lead to formation of reacting factors *in vivo* with the resulting severe injury to the vulnerable skin area. These observations are interesting from the point of view of immunopathology. The state of vulnerability can be induced not only by bacterial filtrates but by active infections as well (15). For this reason it is admissible to assume that the reacting factors formed through the interaction of the antigen secreted by the diseased focus and the actively acquired antibodies may produce, *via* the blood stream, severe injury in vulnerable tissues and organs. This mechanism of production of injury offers

a new working hypothesis for problems concerning relapse of infections, complications, metastatic lesions and generalized spread of disease. There is also suggested the possibility that focal reactions of tuberculin hypersensitiveness may be based on this mechanism. Tuberculin combined with tuberculous antibodies *in vivo* may bring about the formation of reacting factors. These factors would induce severe injury in tuberculous foci, inasmuch as the latter were shown to possess the state of vulnerability necessary for the phenomenon (16).

Bordet observed that reacting factors of heterologous bacterial filtrates were capable of reproducing the phenomenon under discussion at the site of a tuberculous lesion. He called this non-specific allergy. In view of the observations reported here one arrives at an explanation of specific and non-specific allergic manifestations in tuberculous lesions in which both may be an expression of the same mechanism. Thus, in terms of the phenomenon, active infections elicit a state of vulnerability in the diseased tissue. Severe injury then can be induced in this tissue by means of potent heterologous bacterial filtrates, since it has been shown (7) that preparatory and reacting factors of unrelated bacterial species can substitute for each other, provided they are potent (*i.e.*, non-specific allergy). Also injury can be produced in infected tissues by reacting factors formed through the interaction of the injected specific antigen with the actively acquired antibodies of the infected animals (*i.e.*, specific allergy or bacterial hypersensitiveness).

Inasmuch as the interaction of animal protein with the homologous antibody forms reacting factors injurious to tissues made vulnerable by bacterial filtrates or infections, there remains the question of the influence of serum sickness upon the course of infectious diseases.

To recapitulate, the essential mechanism of the phenomenon of local skin reactivity to bacterial filtrates consists in the alteration of tissues by bacterial filtrates or infections whereby they become vulnerable to a variety of toxic principles which act *via* the blood stream. These toxic principles are as follows:

Neutralizable and antigenic exotoxins (true toxins?); toxic factors formed through a colloidal disturbance in the blood stream (anaphylatoxins?); toxic factors resulting from the *in vivo* interaction of non-bacterial antigens with homologous antibodies (true

anaphylaxis?) and toxic factors resulting from the *in vivo* interaction of bacterial antigens with specific antibodies (bacterial hypersensitivity?).

Thus the phenomenon of local skin reactivity to bacterial filtrates must be considered in various fields of immunology. It offers interesting possibilities for correlative studies.

#### SUMMARY

In this paper there is described a new method for the demonstration of antigen-antibody combination, as follows:

A rabbit sensitized a week previously to some animal protein receives a skin-preparatory injection of a potent bacterial filtrate. 24 hours after the skin preparation the rabbit is injected intravenously with the same animal protein. From 4 to 5 hours later there appears severe hemorrhagic necrosis at the prepared skin site.

The incidence of positive results is high. A single sensitizing injection and 1 week incubation period are sufficient. The test is highly specific unless repeated sensitizing injections of large doses of antigen are made. The necessary sensitization can be elicited with minute quantities of animal proteins.

It is also possible to elicit severe reactions in the prepared skin sites of non-sensitized rabbits upon separate intravenous injections of non-bacterial and bacterial antigens and homologous antibodies (*i.e.*, passive transfer).

The relation of the phenomenon of local skin reactivity to bacterial filtrates to various fields of immunology is discussed in the light of the above observations.

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# PROPERTIES OF THE CAUSATIVE AGENT OF A CHICKEN TUMOR

## VII. SEPARATION OF THE ASSOCIATED INHIBITOR FROM TUMOR EXTRACTS\*

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(Received for publication, June 30, 1932)

The presence of an inhibitor or balancing factor associated with the causative agent in a chicken tumor was first indicated by the fact that the tumor-producing activity of the agent was greatly increased when a fraction of the tumor extract or filtrate was removed. This indication was confirmed by the demonstration that certain relatively slow-growing chicken tumors yielded the inhibitor in sufficient concentration to neutralize the tumor agent in the most active extracts (1). It has seemed desirable in the further study of the inhibitor fraction to secure it in greater concentration and freed so far as possible from the many other products contained in the crude tumor extract. The present paper is a report of the first step in this direction.

### *The Release of Chicken Tumor I Inhibitor after Adsorption on Aluminum Hydroxide*

Previous experiments have shown that the most effective way of freeing a tumor extract of the inhibitor was to treat it with aluminum hydroxide (Willstätter Type C) (2), which under proper conditions appears to adsorb the inhibitor factor almost entirely but very little of the tumor-producing agent. The possibility of releasing the inhibitor from the aluminum hydroxide, thus freeing it from some of the impurities, was tested in the following experiments.

*Experiment.*—The material utilized in the experiments consisted of extracts of desiccated Chicken Tumor 1 known to yield sufficient inhibitor to neutralize active tumor filtrates, and to inhibit the growth of a mouse sarcoma (Crocker 180)

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\* This investigation was carried out by means of the Rutherford Donation.

(4); and extracts of tumor desiccates known to be without effect on the chicken tumor or the mouse sarcoma. These materials were prepared by thoroughly extracting 1 gm. of the dry tumor powder with 30 cc. of water. After centrifuging

TABLE I

*Release of Chicken Tumor Inhibitor after Adsorption on Aluminum Hydroxide*

Materials inoculated	No. of inoculations	No. negative	Average size of tumors cm.	Negative per cent
Material from slow-growing tumors				
0.2 cc. active C. T. I filtrate plus 0.2 cc. heated extract slow-growing tumor	19	18	0.6 x 0.4	94.4
0.2 cc. active C. T. I filtrate plus 0.2 cc. heated aluminum supernatant fluid of slow-growing tumor extract	19	1	2.3 x 1.8	5.3
0.2 cc. active C. T. I filtrate plus 0.2 cc. phosphate release from aluminum	19	18	1.1 x 1.0	94.4
0.2 cc. active C. T. I filtrate plus 0.2 cc. water	36	0	2.3 x 1.9	0
0.2 cc. active C. T. I filtrate plus 0.2 cc. M/15 basic sodium phosphate	14	0	2.2 x 1.9	0
Material from rapidly growing tumors				
0.2 cc. active C. T. I filtrate plus 0.2 cc. heated extract rapidly growing tumor	9	0	1.7 x 1.4	0
0.2 cc. active C. T. I filtrate plus 0.2 cc. heated aluminum supernatant fluid of rapidly growing tumor extract	9	0	2.1 x 1.6	0
0.2 cc. active C. T. I filtrate plus 0.2 cc. phosphate release from aluminum	9	0	1.9 x 1.6	0
0.2 cc. active C. T. I filtrate plus 0.2 cc. water	18	0	2.4 x 1.8	0

out the larger particles the supernatant fluid was filtered through filter paper, adjusted to about pH 7.2 and 20 cc. of this was shaken with an equal volume of Willstätter Type C aluminum hydroxide. The aluminum was then separated out

by centrifugation and washed with 10 cc. of distilled water 2 to 3 times. The wash waters were then discarded and the aluminum deposit was shaken up with 10 cc. of M/15 basic sodium phosphate at pH 9, the released material having a final pH of 8. The aluminum was removed and discarded. The eluate was heated at 52° for 30 minutes to inactivate any tumor agent which might have come through.

The tests consisted of the injection of mixtures in equal amounts of the eluate and active tumor filtrate, controlled by the injection of the active filtrate diluted with the heated aluminum supernatant fluid, with water and with M/15 basic sodium phosphate buffer. The inoculations were made with 0.4 cc. of the mixtures intradermally, so that each chicken received the test material and those of the controls as well. In the first group of experiments the extracts were prepared from tumor desiccates known to yield the inhibitor factor and in the second group from desiccates of rapidly growing tumors, known to yield no inhibiting substance. The results of 11 experiments with 152 inoculations are given in Table I.

It is evident from Table I that the inhibiting factor may be adsorbed on aluminum hydroxide and released with basic sodium phosphate. While the experiments were not designed to test quantitative differences between the original extract and the released material, yet they indicate that there is certainly no very great loss in the inhibiting property after adsorption and release. The failure of the heated extracts from the rapidly growing tumor to influence materially the tumor production by active filtrates is in line with previous observations and, as might be expected, there is no evidence of inhibiting action by the released material. That the phosphate in the released fluid is not responsible for the inhibiting action is shown not only by the controls, in which the phosphate was added to the active filtrate, but also by the tests with the released material from the rapidly growing tumor extract.

*Release of Inhibitor from Chicken Tumor X after Adsorption on  
Aluminum Hydroxide*

Chicken Tumor X, a very slow-growing fibrosarcoma, has proved a source of an inhibitor for Chicken Tumor I and Mouse Tumor 180 (3, 4). The inhibitor, judged by previous tests, is less potent than that from Chicken Tumor I, which might be due to the fact that the agent with which it is associated is less vigorous.



*Experiment.*—The technical procedure used in these experiments was the same as that described above. The source of the fluids tested for inhibiting action was extracts of desiccates prepared from Chicken Tumor X, which had required 9 months to a year to reach a large size. Beside the heated full extract, the supernatant fluid after the extract had been adsorbed out with aluminum hydroxide and the material released from the aluminum by treatment with basic sodium phosphate were tested against an active extract of Chicken Tumor I. In each instance the fluids to be tested were heated at 52° for 30 minutes. The controls included the active extract diluted with salt solution and with basic sodium phos-

TABLE II

*Release of the Inhibitor from Chicken Tumor X after Adsorption of Aluminum Hydroxide*

(Based on 8 experiments)

Materials inoculated	No. of inoculations	No. negative	Average size of tumors cm.	Negative per cent
0.2 cc. active C. T. I filtrate plus 0.2 cc. heated extract C. T. X }	21	18	0.9 x 0.8	85.7
0.2 cc. active C. T. I filtrate plus 0.2 cc. heated aluminum supernatant fluid of C. T. X }	13	0	1.9 x 1.6	0
0.2 cc. active C. T. I filtrate plus 0.2 cc. phosphate release from aluminum }	16	14	0.4 x 0.4	87.5
0.2 cc. active C. T. I filtrate plus 0.2 cc. M/15 basic sodium phosphate }	10	0	1.8 x 1.5	0
0.2 cc. active C. T. I filtrate plus 0.2 cc. salt solution }	22	0	2.2 x 1.5	0

phate. The results of the intradermal inoculations, in which each chicken received the control injections and the test inoculations as well, are given in Table II.

The results show conclusively that Chicken Tumor X contains an inhibiting factor capable of neutralizing the agent of Chicken Tumor I, and that the inhibitor is adsorbed by aluminum hydroxide and can be released from this combination. There is no indication of loss in potency of the inhibitor by this manipulation, a finding similar to that noted in the first group of experiments.

## DISCUSSION

There is no doubt that it is possible to adsorb out practically all of the tumor agent from a filtrate with aluminum hydroxide when a large amount of the colloid is used (5). With the proportions used in these and the earlier experiments reported comparatively little of the agent is taken up, but the inhibiting factor seems to be entirely removed and to be recoverable from the aluminum. The amount of inhibitor lost by this treatment has not yet been accurately determined, but certainly effective quantities are present in the eluate.

## SUMMARY

The inhibiting factor present in certain relatively slow-growing strains of Chicken Tumor I and in Chicken Tumor X is adsorbed from extracts of the desiccated tumors by aluminum hydroxide (Willstätter Type C) and can be released in effective quantities from this combination by treatment with basic sodium phosphate.

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# PROPERTIES OF THE CAUSATIVE AGENT OF A CHICKEN TUMOR

## VIII. THE EFFECT OF TESTICLE EXTRACT ON THE RATE OF GROWTH OF CHICKEN TUMOR I\*

BY ERNEST STURM AND F. DURAN-REYNALS, M.D.

*(From the Laboratories of The Rockefeller Institute for Medical Research)*

(Received for publication, June 30, 1932)

The observation that testicle extract markedly enhances the infectivity of certain viruses and bacteria, reported by one of us (Duran-Reynals (1)) has been confirmed and extended by a number of investigators (2). It has been further shown that testicle extract exerts an inhibitory influence on the growth of a transplantable epithelioma of the rabbit (3) and to a less extent on a sarcoma of the mouse (4). It is of interest to know what effect testicle extracts will have on the chicken neoplasms, transmissible by cell-free extracts.

Recently Hoffman, Parker and Walker (5) have published the results of a similar investigation in which they report evidence of the enhancement of the chicken tumor agent by rabbit testicle extract, but none by that from rooster testicle. This evidence is based on two positive experiments, but the same testicle extract was used in both tests. They draw no conclusions as to the nature of the tumor agent from these results, but give the impression that further investigation with the method might lead to some conclusions on the question. During the last 3 or 4 years a considerable amount of data had been accumulated in this laboratory, but had not been published at the time the article referred to appeared. Since our results differed from those reported by the above authors, we deferred publication until additional tests could be made with certain minor modifications in technique suggested by their paper.

\* This investigation was carried out by means of the Rutherford Donation.



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### *Materials and Methods*

The testicle extracts were prepared by removing the glands of rat, rabbit or rooster, trimming them of all adhering tissue and removing the capsule. The tissue was then ground with sand in a mortar along with an equal volume of physiological salt or Ringer's solution. After centrifuging down the heavier particles, the supernatant fluid was decanted, filtered through a Berkefeld V candle and used in the tests.

The testicle extracts of rat, rabbit and rooster and the purified bull testicle extract were tested with (1) the active extract of desiccated Chicken Tumor I, (2) the concentrated Berkefeld filtrate of fresh Chicken Tumor I and (3) suspensions of the tumor cells.

#### *The Effect of Testicle Extract on the Activity of Extracts of Chicken Tumor I Desiccates*

These experiments, both in the method of preparation of the testicle extracts and the location of inoculations, followed closely the technique used in testing the action of the enhancement factor on viruses and bacteria which has been reported by one of us (Duran-Reynals).

*Experiments.*—The tumor extract was prepared by thoroughly extracting 1 gm. of finely powdered desiccate of Chicken Tumor I with 50 cc. of distilled water and adding enough N/10 NaOH to render the solution either neutral or slightly alkaline. A pH of 7.2 to 7.4 was usually maintained in all extractions. The emulsion suspension was thoroughly mixed by passing it in and out of a large hypodermic syringe fitted with a 12 gauge lumbar puncture needle for several minutes. After centrifugation the supernatant fluid was filtered through moistened, coarse filter paper and divided into 5 portions. To each of the first 4 portions was added an equivalent amount of testicle extract to be tested, while to the remaining 5th portion an equal volume of either salt or Ringer's solution was added—the latter to serve for the activity control injections. In some of the tests the testicle extracts were diluted 10 times with Ringer's solution, but this modification of the method had no effect on the results. In consequence all of the experiments are grouped together. The solutions of the testicle and chicken tumor extracts were thoroughly mixed and allowed to stand at room temperature for the length of time necessary to complete the injection. This period usually varied from 20 to 40 minutes. 0.4 cc. of each of the respective mixtures, including the control, was injected intradermally into a normal chicken. By this means each chicken received the 4 test injections and the control for activity. It was noted that the bleb resulting from the intradermal injection of the solutions containing testicle extract flattened in a very few minutes after injection, while those without testicle extract required considerably longer to spread into the surrounding tissues.

Weekly measurements of the resulting tumors were made. The figures on the

size of the tumors recorded for each chicken were the last before the tumors broke through into the subcutaneous tissue or coalesced in the skin. This method in our opinion gives a fair picture of the results. As each bird carried all of the test inoculations as well as the control injections, the variation in susceptibility of individual fowls and in the activity of the different tumor extracts is minimized. The chickens used in the tests were of the Plymouth Rock type, adults of uncertain age, bought in the open market. Fresh extracts were used in each group. The outcome of 4 experiments, based on 72 inoculations, is given in Table I.

There is no evidence of enhancement of the chicken tumor agent by the various testicle extracts with the methods used in these experiments. Nor do we find any inhibiting action, with the possible exception of the tests with purified bull testicle; but the numbers here are too small to be significant. Furthermore, at no time during the course of the experiments was there any material evidence of enhancement or inhibition in the development of the tumors.

TABLE I  
*The Action of Testicle Extract on Chicken Tumor I Extracts*  
(4 experiments)

Chicken Tumor I extract	No. of inoculations	No. of tumors	Average size of tumors
			<i>cm.</i>
Rat testicle extract.....	20	20	2.5 x 2.0
Rabbit testicle extract.....	8	8	2.3 x 1.8
Rooster testicle extract.....	16	16	2.5 x 2.0
Purified bull testicle extract.....	4	4	1.6 x 1.1
Salt solution (control).....	24	24	2.3 x 1.9

*The Effect of Testicle Extract on the Tumor Production by a Concentrated Berkefeld Filtrate of Chicken Tumor I*

As a further test of the possible effect of testicle extract on the chicken tumor agent, filtrates of fresh tumor extracts were utilized in the next group of experiments instead of the extracts of tumor desiccate, which were the source of the agent in the preceding group.

*Experiments.*—Berkefeld filtrates were prepared by grinding 25 gm. of mashed chicken tumor tissue in a mortar along with a quantity of sterile sand, diluting with 500 cc. of distilled water and adding N/10 NaOH to bring the pH of the solution to 7.2–7.4. The solution was shaken for 20 minutes in a stoppered flask, centri-



fuged and the supernatant fluid passed through Berkefeld V candles under 25 lbs. of air pressure. 300 cc. of the filtrate was collected and concentrated to 40 cc. in alundum thimbles lined with 8 per cent collodion membranes. The testicle extracts were prepared in the same manner as in the previous experiments.

For the test, 0.4 cc. of mixtures of equal parts of the concentrated filtrate and testicle extracts was injected intradermally in chickens. For the control of activity an equal amount of the concentrate diluted with Ringer's solution was injected into each chicken. Fresh testicle extracts and tumor filtrates were prepared for every experiment. The results of two groups of tests are given in Table II.

While the number of fowls inoculated in this group is not large, yet they show a uniform lack of any effect of the testicle extracts on the size of tumors produced by tumor filtrates.

TABLE II  
*The Action of Testicle Extract on Fresh Concentrated Berkefeld Filtrate of  
Chicken Tumor I*  
(2 experiments)

Concentrated Berkefeld filtrate of Chicken Tumor I	No. of inoculations	No. of tumors	Average size of tumors
			cm.
Rat testicle extract.....	6	6	1.8 x 1.3
Rooster testicle extract.....	6	6	1.8 x 1.6
Salt solution (control).....	6	6	2.1 x 1.5

*The Effect of Testicle Extract on the Size of Tumors Resulting from the  
Inoculation of Tumor Cells*

As stated above, testicle extract definitely inhibits the growth of a transplantable rabbit tumor and to a less extent of a certain transplantable mouse tumor. In these instances the tumors are transferred only by cells. To parallel these experiments more closely we have tested the effect of testicle extract on the size of tumors produced by the chicken tumor cells.

*Experiments.*—The chicken tumor cell suspensions were made by taking 1 cc. of freshly mashed chicken tumor tissue, passing it through a fine wire gauze mesh under slight pressure and suspending the cells in 5 cc. of Ringer's solution. For the injection a mixture was made of equal parts of the cell suspension and the respective testicle extracts. The total volume injected into each area of a normal chicken was 0.4 cc. The results of these experiments are shown in Table III.

From the figures given in Table III there seems to be no enhancement of the growth rate of tumors resulting from mixtures of chicken tumor cells with testicle extract from four different species.

*The Effect of Testicle Extract on the Development of Tumors in Young Fowls*

Hoffman, Parker and Walker (5), in their experiments referred to above, used 8 weeks old chicks and reported enhancement with extracts of rabbit testicle (2 experiments with the same extract), but none with that from the rooster. In order to check the possibility that the age of the fowl affects the results our experiments have been repeated using chicks 6 to 8 weeks old.

TABLE III  
*The Action of Testicle Extract on Chicken Tumor I Cells*  
(3 experiments)

Chicken Tumor I cell suspension	No. of inoculations	No. of tumors	Average size of tumors cm.
Rat testicle extract.....	6	6	2.0 x 1.4
Rabbit testicle extract.....	14	14	2.2 x 1.6
Rooster testicle extract.....	3	3	2.0 x 1.4
Purified bull testicle extract.....	2	2	1.9 x 1.5
Salt solution (control).....	12	12	2.3 x 1.5

*Experiments.*—The tests were confined to the effect of rat and rabbit testicle extracts, but these were tested on both tumor cells and extracts of desiccated tumor, the inoculations being made intradermally. The conditions of the experiments were the same as in the preceding groups, except that young chicks were used. The results of these experiments, with fresh extracts for each experiment, are given in Table IV.

A further experiment was carried out with 8 weeks old chicks, in which a mixture of chicken tumor cells and rat testicle extract was injected intramuscularly in one side, and in the other the tumor cell suspension appropriately diluted for control. The measurements of the resulting tumors suggested some enhancement in 3 of the 4 fowls. They were killed at the height of the experiment and the true measurements made at autopsy showed no material difference between the two sides. This was verified by the weight of the two tumors in the one chick showing the greatest difference in the crude measurements.

From these last tests it seems that there is no evidence of enhancement of tumors by testicle extract in young chicks, thus confirming the results we had obtained in adult birds.

TABLE IV  
*The Action of Testicle Extract on Chicken Tumor I*  
*8 Weeks Old Chicks Injected*  
(3 experiments)

Material injected	No. of inoculations	No. of tumors	Average size of tumors
			cm.
Chicken tumor cells with:			
Rat testicle extract.....	4	4	3.3 x 2.5
Rabbit testicle extract.....	4	4	3.2 x 1.9
Salt solution (control).....	8	8	3.2 x 2.3
Chicken tumor extract with:			
Rat testicle extract.....	7	7	2.3 x 2.2
Rabbit testicle extract.....	7	7	2.1 x 1.6
Salt solution (control).....	14	14	2.2 x 1.7

#### DISCUSSION

With testicle extract we have a factor or factors which augment the infection of viruses and bacteria on the one hand, and on the other seem to retard the growth of certain transplantable tumors. With this evidence it might be supposed that the action of testicle extract on the chicken tumor would give suggestive information as to the nature of the causative agent. However, since the testicle factor enhances infective agents (6) by increasing tissue and cell permeability, and since it enhances the action of certain toxins (7), it would appear that no deduction could have been drawn as to the nature of the chicken tumor agents even if the experiments had shown a definite enhancement. As a matter of fact we have found no evidence of definite enhancement or inhibition with the various testicle extracts investigated. There may be some simple explanation in this failure such as the structure of the fowl's skin, but this does not seem likely since the ability of the testicle extract to bring about an increase in the area of spread of injected materials is just as evident in the chicken

skin as in that of the mammal. We see no immediate explanation of the difference in the results of our experiments and those of Hoffman, Parker and Walker.

#### SUMMARY

Extracts prepared from the testicle tissue of the rat, rabbit, fowl or bull, injected together with extracts of Chicken Tumor I or with cells of this tumor, showed no definite effect of either enhancement or inhibition as concerned the resulting tumors.

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# STUDIES ON THE PHYSIOLOGICAL EFFECTS OF FEVER TEMPERATURES

## I. A DESCRIPTION OF A SERIES OF CONSTANT TEMPERATURE WATER BATHS FOR THE DETERMINATION OF THE THERMAL DEATH TIME OF BACTERIA\*

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In order to study the *in vitro* thermal death time of bacteria as well as that of tissue and tumor cells, it was necessary to have a series of constant temperature water baths to cover the range of fever temperature in man from 37–42°C. The purchase of many single units would have been expensive. The construction of such baths also seemed difficult and expensive, but after a consultation with the various members of the Departments of Bacteriology, Physiology, and Vital Economics of this school, it was decided to construct six baths as a unit in one table. This would enable us to use a single counter-shaft and motor for the stirrers and make the controlling equipment compact and simple.

### *Construction*

Six ordinary galvanized iron boxes were obtained from the Rochester Can Co. These were 55 x 41 cm. at the top, tapering to 49 x 34 cm. at the bottom, and 25 cm. deep. They were thoroughly painted inside with aluminum bronze lacquer. A solid trough-like table (Fig. 1) was built to support these boxes flush with its upper surface. Each was separated from the other by a thin board partition. A space of 2 cm. was left between the top of the metal boxes and the surrounding walls, and the space filled with ground cork. The bottom of the metal boxes

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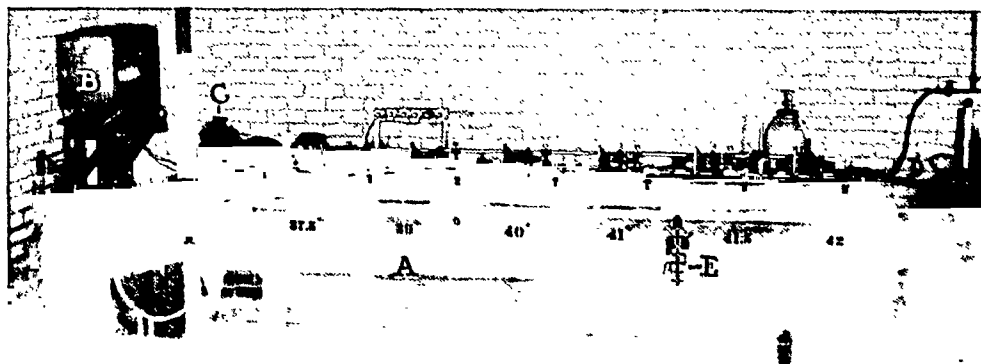


FIG. 1. Series of six constant temperature baths for studying the thermal death time of bacteria. *A*, table supporting baths; *B*, recording galvanometer; *C*, rotating switch; *D*, siphon arrangement to maintain water levels; *E*, dental film holders to which are attached the labelled, sealed vials. Used to immerse vials in baths.

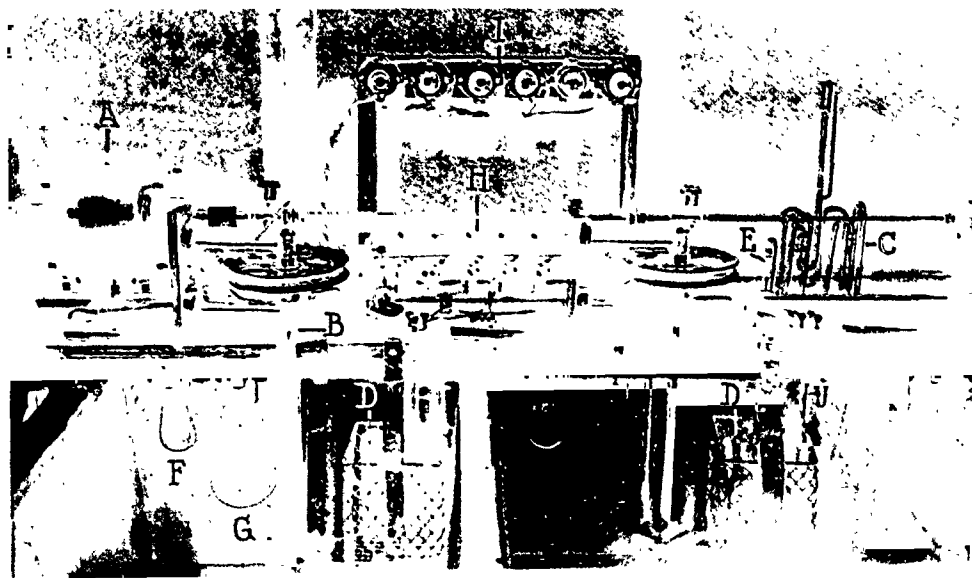


FIG. 2. Detail of two of the series of six baths. *A*, motor; *B*, stirrer; *C*, thermo-regulator—disconnected showing type and construction; *D*, thermoregulator—installed within basket that affords it protection; *E*, adjuster screw for thermo-regulator; *F*, continuous heater lamp; *G*, intermittent heater lamp; *H*, series of relays that operate heater lamps; *I*, neon pilot lamps; *J*, resistance thermometer.

rested directly on the tight 2 cm. ( $3/4$  inch) flooring of the table, it being found unnecessary to insulate further the under surface of the metal boxes. Wooden strips were then placed over the metal box edges to hold them and the cork packing in place. A 4 cm. plank about 25 cm. (10 inches) wide was then fitted the full length of the back of the table so that its center rested on the posterior rim of the metal boxes. It thus projected over the back edge of the table, as well as some 8 cm. over the baths. This plank supported the motor, the bearings and shaft, the bearings and support for the stirrers, the thermoregulators and their relays, all wiring, the heater lamps, the resistance thermometers, and a water-levelling device (Fig. 1). The next and most important step at this point was to waterproof all wooden surfaces and joints with hot paraffin to prevent warping. The hot paraffin was spread on quickly with a large brush until the wood was well saturated.

The stirrer supports and bearings were mounted so that a 12 cm. paddle wheel occupied about the middle third of each bath. The paddle wheel was cut from a brass disc about 1 mm. thick. This was mounted on the end of a 13 mm. ( $1/2$  inch) brass shaft between two washers. The shaft passes up through the bearings and a pulley wheel, which is held in place vertically by a bushing and set screw. The pulley wheel and bearings were stock products, common in laboratory supplies. The bearings for the pulley wheel were bolted to right angle braces, which were firmly screwed near the front of the plank mentioned above. Oil holes were drilled in the top of the stirrer shaft to reach the two bearings and the bushing. These holes were packed with felt in order to allow a slow, steady supply of lubricant. The 16 mm. ( $5/8$  inch) main driving shaft and its bearings were mounted on a piece of 13 mm. ( $1/2$  inch) thick, 52 mm. (2 inches) wide, and  $2\frac{1}{2}$  m. (98 inches) long, flat, cold-rolled steel which was screwed on the back half of the plank. A stock adjustable, floating type of bearing was found to be most satisfactory. Each had its own grease cup. Standard 52 mm. (2 inch) pulleys with 13 mm. ( $1/2$  inch) grooves were placed in the proper place opposite each stirrer pulley. A one-third horse power type S.R., 1150 R.P.M., A.C. motor, protected by a thermal cut-out, was mounted at one end of the plank and joined by a flexible coupling to the main shaft. Endless leather belts 0.65 cm. ( $1/4$  inch) in diameter were found to be most satisfactory for running the stirrers. The belts were kept pliable by the application of belt dressing. Two standard keyless, porcelain socket receptacles were mounted on the under side of the plank on the left of the stirrer in each bath for the carbon filament heater lamps. A 10 watt carbon filament lamp, used as a pilot heater, was placed in one socket, while the other socket contained a 60 or 120 watt carbon filament lamp, which is used as an intermittent heater and is controlled by the relay and thermoregulator of the individual bath. A bank of six telephone relays, modified to operate on 12 volts D.C. was mounted on the left end of the plank near the motor. A 12 volt battery energizing the relay circuit is kept on a floating charge. The connections are shown in the wiring diagram (Fig. 3).

The thermoregulators are hung in a wire basket attached to the plank at the left of the stirrers. They consist essentially of a glass capillary tube and bulb,



a side arm with platinum wire fused in the wall to make contact with mercury in the capillary. Attached to the bulb is about 1 m. of 0.5 cm. glass tubing bent into a series of twelve compact loops which are filled with toluene. The bulb is filled with just enough clean mercury so that when the regulator is placed in a bath at the desired temperature the mercury ascends about half way up the capillary. The junction between the toluene and the mercury should occur in about the middle of the bulb so that a little tilting is possible during the mounting process without allowing the toluene to enter the capillary. The side arm is filled with mercury in which is immersed one of the connections to the relay, the circuit being completed through the mercury in the capillary and the tempera-

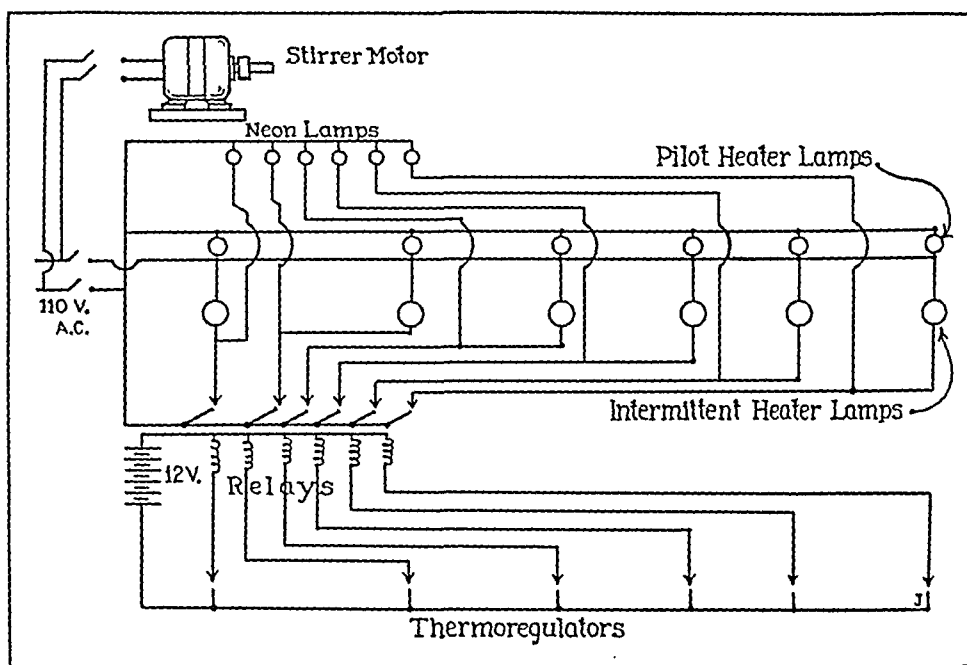


FIG. 3. Wiring diagram for constant temperature baths, with the recording thermometer circuits omitted.

ture-adjusting screw. The top of the regulator with its adjustable contact and side arm projects through a hole in the plank. The temperature is adjusted by means of the movements of a 6-32 screw 5 cm. long, to which is soldered a 6 to 8 cm. length of No. 28 (B. and S.) nichrome wire. This wire passes down into the 1 mm. capillary of the regulator to make contact with the head of the mercury column. The thermoregulators require about 17 turns of the screw to change the temperature of the bath  $0.1^{\circ}\text{C}$ . The glass blowing for the thermoregulators was done by Mr. W. Latchford of the Department of Physiology.

When the relay is activated so as to shut off the intermittent heater lamp, a small neon bulb is lighted (Fig. 3). Thus the rate at which the heater lamps flash on and off can be visualized and a check kept upon the operation of the baths.

The temperature of the baths with covers does not vary more than  $0.002^{\circ}\text{C}$  determined by a Beckman thermometer. Thermocouple measurements at various locations in the bath show that the temperature is uniform.

A resistance thermometer, encased in a brass tube, is placed beside the thermometer of each bath. Each one of the six resistance thermometers is connected successively every 2 minutes with a registering galvanometer by means of a rotating switch (Fig. 1) driven by a slow speed motor.

While this recorder cannot show extremely small fluctuations of temperature, it does permit the observation of variations of  $0.1^{\circ}\text{C}$ . or over, and allows a check on the constancy of the temperatures when the baths are allowed to run during the night without attendance.

There is an intercommunicating siphon of 1 cm. brass pipe between all baths, with an outside level maintained in a sink by a constant drip and overflow. By means of stop-cocks it is possible to fill or empty the baths, or maintain a constant water level, thus compensating for losses by evaporation.

#### SUMMARY

A series of six water baths in one unit, set at  $37^{\circ}$ ,  $39^{\circ}$ ,  $40^{\circ}$ ,  $41^{\circ}$ ,  $41.5^{\circ}$ , and  $42^{\circ}\text{C}$ ., for the purpose of studying the thermal death time of bacteria and of body tissues has been described.

We desire to thank Mr. Ernest Wolfram for his excellent technical assistance.



# STUDIES ON THE PHYSIOLOGICAL EFFECTS OF FEVER TEMPERATURES

## II. THE EFFECT OF REPEATED SHORT WAVE (30 METER) FEVERS ON GROWTH AND FERTILITY OF RABBITS\*

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PLATES 34 AND 35

(Received for publication, June 30, 1932)

The development of the short wave vacuum tube radio oscillator has opened many new fields for biological research. The study of the effect of short wave high frequency radiation (4 to 300 m.) on bacteria and their products, on plant and animal cells, as well as upon normal and diseased animals, is engaging the attention of many investigators. During the last 5 years approximately thirty reports on the biological effects of high frequency radiation have been published. A recent survey of this work was made by Szymanowski and Hicks (1). The production of therapeutic fevers by placing patients in a high frequency electrostatic field is still an experimental procedure, but the clinical work thus far done indicates that this procedure may have unusual possibilities. Before this method of treating disease in man can be accepted for clinical use sufficient proof must be established to demonstrate that there are no injurious results. Three possible biological effects of such radiation must be considered: first, the general heating effect; second, the possible selective heating due to special dielectric properties of different tissues at certain frequencies (principally above 60,000 kilocycles); third, a possible biological influence due to factors other than the temperature elevation of the cell. The latter is difficult

\* This work was made possible by grants from the Research Laboratory of the General Electric Co. and from the Rockefeller Foundation.

to demonstrate because complicated arrangements are necessarily required to eliminate the heating factor. Nevertheless, Szymanowski and Hicks (1) believe they have succeeded in attenuating bacterial toxins by short wave radiation without the development of heat.

### HISTORY

In 1893 d'Arsonval (2) reported that high frequency currents failed to stimulate tissues but only gave a sensation of heat. He was able to elevate the body temperatures of animals and with such currents to stimulate their metabolism. Schereschewsky (3) working with a vacuum tube oscillator studied the effect of short wave radiation on mice. He determined that wave lengths from 3.8 to 15 m. (80,000 to 20,000 kilocycles) had the most marked lethal effect on mice. He noted a rise in their body temperature but his explanation for the cause of death was that an electromechanical vibration destroyed the living cells of the body. He believed that this was a specific effect, especially since the rectal temperature of dead mice could be raised only from 0.1–0.7°C., while in the same length of time the rectal temperature of living mice was elevated from 5–6°C. Christie and Loomis (4) repeated similar work on mice, but maintained that the lethal effects were due to the heat generated by the high frequency current which was induced in the mice. They were unable to elicit any evidence of a specific effect. McKinley (5) made interesting observations on heating the adult and larval form of a beetle, *Tenebrio molitor*, with a short wave oscillator. The adult was killed in 1 minute and 19 seconds, while it took 7 minutes and 38 seconds to kill the larva. He explained this difference by the selectivity of the waves for nervous tissue which is abundant in the adult while the larval form is almost devoid of it. An exposure of 2 minutes and 1 second was required to kill the adult grasshopper, *Melanoplus femur-rubrum*, while the nymph of this same species was killed in 2 minutes and 15 seconds. The length of time required to kill these two forms was practically the same due to the similarity of their nervous structures. Therefore, McKinley postulates a specific effect or selectivity of the short waves apart from the elevation of internal temperature which he believes to be only a by-product of such radiation.

Knudsen and Schaible (6) studied the effect of fevers produced by short wave radiation on 28 white rats. They were heated to an average rectal temperature of 40.5°C. from 30 minutes to an hour, 5 times each week from 8 to 13 weeks. They state that it was difficult to keep the rats at any set temperature, and that during one treatment the temperature of five rats was elevated from 43.3–43.9°C., resulting in the death of one of the animals. They conclude that a temperature of 40.5°C. does not appear to retard the growth of rats when exposed for short daily intervals of  $\frac{1}{2}$  and 1 hour. They state that the reproductive organs of the male and female were not appreciably affected, and that no abnormal pathologic lesions were produced. However, it is also stated that a histological examination

of the male testes showed a retardation of spermatogenesis with exfoliation of the germinal epithelium and a proliferation of the Sertoli cells.

During the last 4 years we have studied the effect of short wave fevers on normal and diseased white mice, white rats, guinea pigs, rabbits, and dogs. We (7) have likewise observed the effect on man of such fevers when used for therapeutic purposes. It has been our general observation that no ill effects occurred except occasional slight superficial burns, unless too high a fever was produced for the species of animal irradiated. In 1929, as a preliminary study, we exposed a series of male and female guinea pigs at irregular intervals, producing a fever with a maximum temperature from 41–42°C. During the time they were receiving such treatments they were bred. The heatings were continued during their pregnancies which terminated normally. No apparent injury occurred to the fetuses *in utero*, since there were born just as large and normal litters with birth weights similar to those from untreated females. Although our impression at that time was that the fever-treated group was somewhat larger and sleeker than the untreated males and pregnant females, we did not have enough observations to be conclusive. Another difficulty we encountered was the inability to establish uniform fevers. This had the same experience when rats and mice were irradiated. This was apparently due to the fact that such small experimental animals were not able to adjust themselves to their ineffective temperature-regulating mechanism. The small body mass of such experimental animals must also be considered. Because of this preliminary observation on guinea pigs and the relative importance of determining the effects of short wave fevers on metabolism and fertility, a similar but more detailed study was made on twenty-four rabbits, including their young. Rabbits were used for this work because of their larger size and the fact that more uniform fever temperatures can be maintained in them than in the smaller species of experimental animals.

#### *Experimental Method*

This procedure was designed to determine whether any gross abnormalities in growth or fertility occur from the continual use of short wave fevers produced in an electrostatic field (10,000 E.S.U.).

The second and third generations were observed for any possible developmental changes resulting from injury to the gonads or to other organs during fetal life. Such effects might result from the increased heat of the fever or from some inherent specificity of the short radio waves.

Twenty-four healthy immature rabbits born to normal, large, adult Belgian hares were chosen from our stock. The mates of six litters, labelled A, B, C, D, E, and F respectively in the order of the kindling date of the litter, consisting of twelve bucks and twelve does, were divided into two groups. The first group contained ten controls, while the second was comprised of fourteen rabbits to be given fever treatments. Since the number and sexes in each litter varied, it was necessary to make an arbitrary division of the sexes and matings in the control and treated groups. Wherever possible litter mates of the treated groups were paired and treated together, assuming that the maximum effect would thus be produced in the young born to them, providing any changes resulted from this form of radiation. In one case a treated buck (No. 11-84) was bred to two control females (Nos. 11-85 and 11-86). The matings and data concerning the number and frequency of the fevers appear in Table I.

The technique of irradiation was as follows: The rabbits were exposed in a wooden box (orange crate) in the electrostatic field of the high frequency oscillator operating at a frequency of 10,000 kilocycles (30 m.). An orange crate, in addition to being inexpensive, offers both ventilation and drainage and the wood, being a good dielectric, does not heat to any great extent. The irradiation was supplied by two oscillators in a Hartley circuit. In one a push-pull circuit was used, while the other gave only half-wave rectification. The d.c. plate voltage on the oscillating tubes was from 1,800 to 2,000 volts, and the plate current was from 2 to 4 milliamperes. The high frequency waves oscillated between two aluminum plates (14 x 20 inches) set upon the cabinet, producing an electrostatic field in which the animals were heated. The high frequency current in the leads to these plates varied from 2 to 4 amperes, depending upon the number and size of the animals in the field, as well as on the distance between the plates. The output of the machine was adjusted in each irradiation to the optimum heating field strength, no set values except frequency being possible under the present conditions. One of the machines was kindly loaned to us by Dr. W. R. Whitney of the General Electric Research Laboratory, where it was designed. The other was built by Mr. F. W. Bishop of our laboratory staff. Both machines operate at the frequency stated above. Oscillation is indicated by the glowing of a neon bulb set near one of the high frequency leads.

The fever treatments were begun on litter mates at ages varying from 29 to 171 days and continued either 2, 3, or 5 times per week until the pregnancy terminated. The bucks likewise were given no more fevers after their mates had kindled. The rabbits in Litters C, D, E, and F were not treated for about 1 month during

TABLE I

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TABLE I  
Effect of Repeated Short Wave Irradiation on Fertility and Growth of Rabbits

Rabbit No.	Sex	Litter	Mated with Rabbit	Age at beginning of treatment	No. of treatments	Duration of treatment	Maximum temperature	Age of rabbits at beginning of treatment	No. in litter	Weight of litter	No. raised	Weight at beginning of treatment	Weight at 1 year	Gain in weight
11-16	F.	A	11-20	123	3	22	41.9	331	6	244	0	1662	2105	21.04
11-18	M.	A	Normal F.	123	Control	—	—	—	—	—	—	1882	2200	14.45
11-20	M.	A	11-16	123	3	26	41.7	—	—	—	—	1725	1913	9.82
11-15	M.	B	11-17-9	171	3	22	41.4	—	—	—	—	2344	2600	9.84
11-17	F.	B	11-15	171	Control	—	—	—	—	—	—	2400	3054	21.41
11-19	F.	B	11-15	171	3	26	41.6	239	7	301	3	2400	3054	21.41
11-83	F.	C	11-84	80	3	36	41.9	251	6	280	0	2364	2980	20.67
11-84	M.	C	11-83-5-6	80	3	36	42.1	183	7	162	0	1099	3500	68.60
11-85	F.	C	11-84	80	Control	—	—	—	—	—	—	985	2915	66.20
11-86	F.	C	11-84	80	Control	—	—	—	—	—	—	1160	3415	66.02
11-87	M.	D	11-88	46	—	—	—	—	—	—	—	1045	3300	68.33
11-88	F.	D	11-87	46	2	30	42.4	—	—	—	—	555	3910	85.80
11-89	F.	D	11-89	46	2	31	42.3	3	123	3	500	3900	87.18	
11-90	M.	D	11-90	46	5	67	42.0	6	351	5	452	3945	88.54	
11-91	M.	D	11-89	46	5	67	42.0	—	—	—	585	4289	86.33	
11-92	M.	D	11-97	46	Control	—	—	—	—	—	581	3290	82.34	
11-93	F.	E	11-08	44	Control	—	—	—	—	—	400	—	—	—
11-94	F.	E	11-08	44	2	31	207	3	158	0	442	4185	89.44	
11-95	F.	E	11-06	44	2	39	255	8	279	0	568	3870	85.32	
11-96	M.	E	11-95	44	Control	—	—	6	250	0	476	3850	87.63	
11-97	F.	E	11-91	44	Control	—	—	5	185	0	533	3700	85.59	
11-98	M.	F	11-03-4	29	39	74	42.3	—	—	—	540	3150	84.34	
11-99	M.	F	Normal F.	29	—	—	—	—	—	—	353	4800	92.65	
12-00	M.	F	Normal F.	29	—	—	—	—	—	—	322	4820	93.31	

Died 83 days of coccidiosis

Died 255 days of pneumonia



an epidemic of diarrhea due to coccidiosis. This occurred in the controls and treated rabbits alike when they were about 90 days old. On the day of the first treatment all of the rabbits in a litter were weighed and thereafter at weekly intervals. Rectal temperatures were taken at the beginning and at 15 minute intervals during the period of the exposure and afterward until the temperature of the rabbit had returned to normal. The current was shut off and the animals were removed from the field to make these observations. At each treatment we endeavored to produce a rectal temperature with fastigium of  $41.5^{\circ}\text{C}.$ , but occasionally the temperature varied from  $41\text{--}42^{\circ}\text{C}.$  The highest temperatures measured during the series of irradiations are recorded in Table I, and it will be observed that in two instances temperatures of  $42.4^{\circ}\text{C}.$  were reached.

The exposure of the animals repeatedly over a long period of time necessitated care to prevent burns from contact with the aluminum plates. It is important not to allow the urine to wet the feet of the rabbits. It contains enough salts to make an excellent condenser, and the concentration of eddy currents in the liquid is sufficient to cause severe burns. While the position of the animal between the aluminum plates makes some difference in the rate of heating, the movement of the animal in the crate is usually sufficient to make local overheating negligible during the treatment.

When the rabbits were young it was possible to irradiate four at a time in the compartments of an orange crate, but as they grew larger mated pairs were heated to maintain similar conditions in both sexes. The control rabbits were likewise brought to the treatment room each day with the animals to be treated. Their temperatures were taken several times, as were those of the irradiated rabbits, in order to handle the former animals as much as the treated animals.

When pregnancy terminated the young in each litter were counted and weighed, and, if they survived, observations were made on their growth and fertility until a third generation was born. The litters comprising the third generation were also counted, weighed, and observed for survival and growth.

Pairs were kept together until pregnancy was definitely established, after which the rabbits were separated. All rabbits were otherwise kept in separate cages and fed on a stock diet of an abundance of whole oats. In the summer months fresh cut green alfalfa was fed daily in addition, while during the rest of the year dried alfalfa and chopped cabbage were fed on alternate days. Fresh water was always available in the cages. All of the animals were fed at the same time each day.

#### EXPERIMENTS

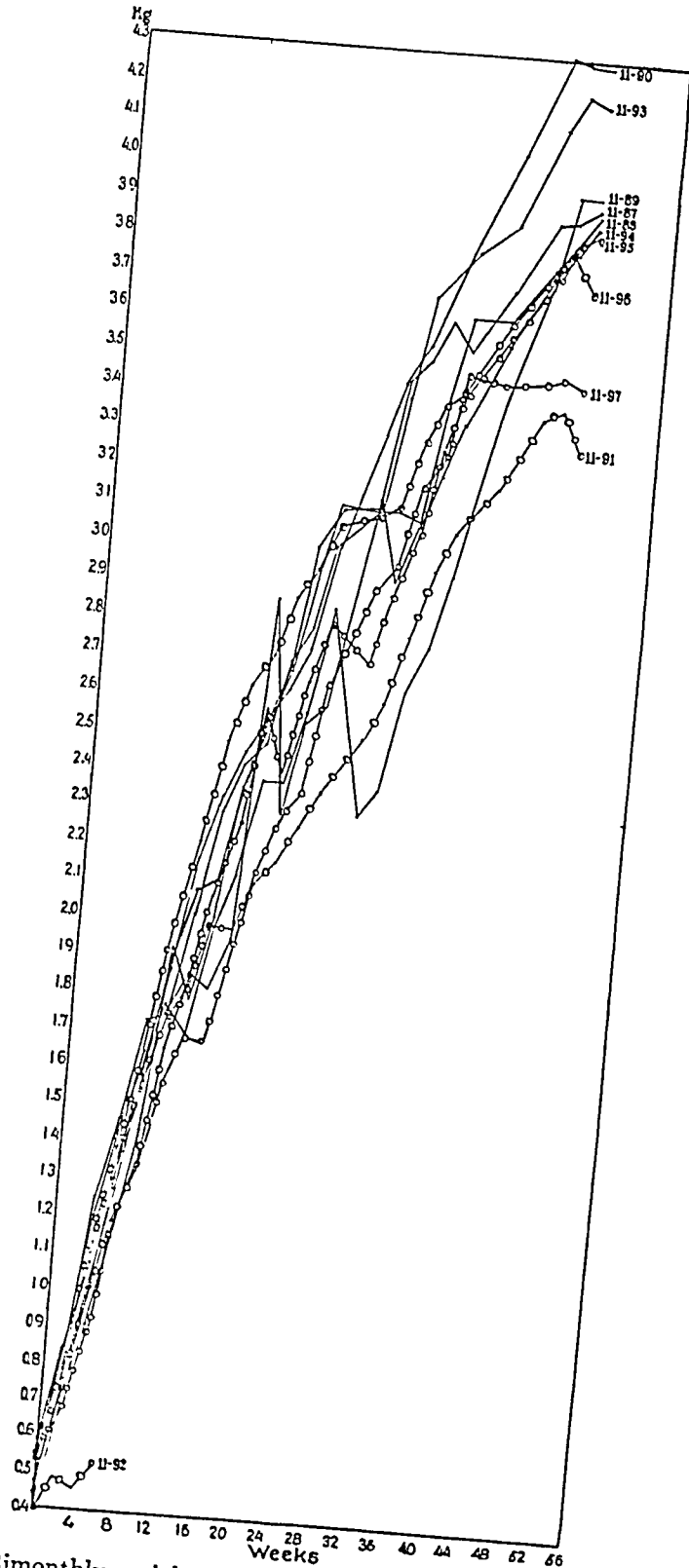
*Experiment 1. Litter A.*—Rabbits 11-16 female and 11-20 male were paired. Beginning at 123 days of age they were given 3 short wave fevers each week with a total of 22 and 26 exposures respectively, with a total amount of  $23\frac{1}{2}$  and 33 hours irradiation. At the age of 331 days the female gave birth to six normal, living young totalling a weight of 244 gm. She refused to nurse them and raised none of the litter. The other male in this litter, No. 11-18, was used for a control.

*Experiment 2. Litter B.*—Rabbits 11-15 male and 11-19 female were paired and beginning at 171 days of age were given 3 fevers each week. The male received 22 exposures, while the female had 26. The total number of hours irradiation was  $24\frac{1}{2}$  and  $32\frac{1}{2}$  respectively. At 251 days of age No. 11-19 had a litter of six normal, living young totalling a birth weight of 280 gm. She gave them no care and all died within 3 days. A female, No. 11-17, was kept as a control. She was bred to Rabbit 11-15, the same male that was used to mate the fever-treated female in this litter. At 239 days of age she bore seven normal, living young totalling a weight of 301 gm. Three of the seven were raised.

*Experiment 3. Litter C.*—Rabbits 11-83 female and 11-84 male, beginning at 80 days of age, were given fever treatments 3 times weekly. A total of 36 fevers were produced, the female receiving a total exposure of  $75\frac{1}{2}$  hours while the male received a total of  $68\frac{1}{2}$  hours. The female conceived and gave birth to a litter of seven rabbits with a total litter weight of 162 gm. Parturition occurred during the night and when found in the morning only one of the litter was living. It died 3 hours later. Two females, Nos. 11-85 and 11-86, in this same litter were used for controls. They were bred to Rabbit 11-84. They conceived and bore normal litters of nine and eight weighing 288 and 256 gm., at 223 and 182 days of age respectively. Neither female raised any of her young. Rabbit 11-85 killed and ate all of her young within 24 hours. All of the litter born to No. 11-86 were living. The mother destroyed three within 4 hours after birth and the remainder within 48 hours.

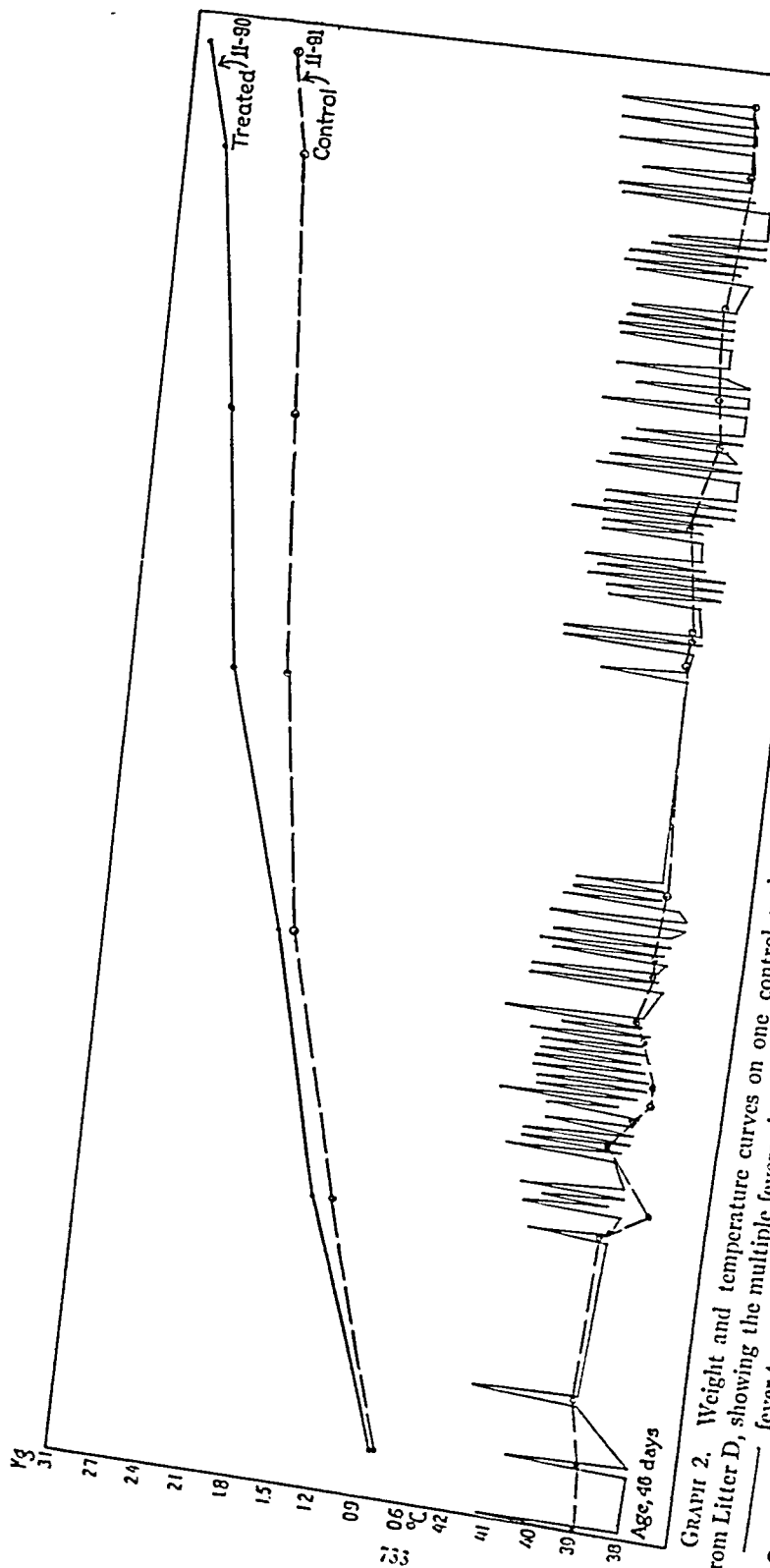
*Experiment 4. Litter D.*—Rabbits 11-87 male and 11-88 female were paired and given biweekly, beginning at 46 days of age, 30 and 31 fevers totalling  $51\frac{1}{2}$  and  $55\frac{1}{2}$  hours irradiation respectively. At 210 days of age the female gave birth to three young totalling 123 gm. in weight. All of the young were raised to maturity. Rabbits 11-89 female and 11-90 male of this same litter were paired. They were given short wave fevers 5 times each week. 67 fevers were given each, totalling  $136\frac{1}{2}$  and  $135\frac{1}{2}$  hours respectively. At 204 days of age the female (No. 11-89) had a litter of six with a total birth weight of 351 gm. Five of these were raised to maturity, the sixth dying 1 week after birth from an unknown cause. Two males, Nos. 11-91 and 11-92, were used for controls. Rabbit 11-92 died at the age of 83 days from coccidiosis. The other control matured normally. The growth curves of the treated and control rabbits in this litter are seen in Graph 1.

*Experiment 5. Litter E.*—Rabbits 11-93 and 11-94 females were bred to Rabbit 11-98 male (Litter F) and treated biweekly 31 and 39 times, totalling  $57\frac{1}{2}$  and  $78\frac{1}{2}$  hours respectively. The first fever was given at 44 days of age. At 207 days of age No. 11-93 gave birth to three normal, living young totalling a weight of 158 gm. Two of the litter died in 2 days and the third on the 4th day after birth. Rabbit 11-94 had a litter of eight rabbits weighing 279 gm. at birth. Neither female raised any of her young. The three other rabbits in Litter E served as controls. Female 11-95 was bred to Male 11-96, and Female 11-97 was bred to Male 11-91 (Litter D). At 172 days of age Rabbit 11-95 gave birth to six young totalling a weight of 250 gm. All were dead except one when found



GRAPH 1. Bimonthly weight curves of control and of short wave (30 m.) fever-treated rabbits.

— fever-treated.  
 ○—○ controls.



GRAPH 2. Weight and temperature curves on one control male (No. 11-91) and his litter mate (No. 11-90), a fever-treated rabbit, both from Litter D, showing the multiple fevers given over a period of 158 days.

— fever-treated.  
 O — controls.

and the latter died a few hours later. Rabbit 11-97 gave birth to five young totalling a weight of 185 gm. when she was 216 days old. The mother refused to nurse the litter and to care for them, which resulted in their death within 48 hours after birth.

*Experiment 6. Litter F.*—The three males in Litter F, Nos. 11-98, 11-99, and 12-00, were used as follows: Rabbit 11-98, as mentioned above, was bred to the two treated females in Litter E (Nos. 11-93 and 11-94). Rabbit 11-98 was given 39 fevers biweekly, totalling  $74\frac{1}{2}$  hours irradiation. Rabbit 11-99 was used for a control, while No. 12-00, also a control, died at the age of 255 days from pneumonia.

### RESULTS

It will be noted from Table I and from the growth curves of the controls and of the treated rabbits in Graph 1, that no evidence of injury was caused by repeatedly irradiating rabbits in an electrostatic field of short radio waves (30 m.), thereby elevating their body temperature from 41–42°C. for a considerable length of time. Weekly weights were observed until they had attained their maximum weight at maturity (age when body weight maintained practically a uniform level). In the majority of cases the rabbits exposed to repeated short wave fevers gained a greater maximum weight than did the controls. However, the treated bucks did not attain as great a weight as that of the treated does 3 or 4 months after kindling. The coats of the irradiated group of rabbits were smoother and sleeker than those of the non-irradiated group. In practically every instance one not familiar with the experiment could easily differentiate the two groups by the better general appearance and size of the fever-treated rabbits. The latter appeared more vigorous, healthier, and better nourished (see Fig. 1).

Regardless of the appearance of the two groups, the most reliable information is the percentage of gain in weight. It is difficult to make comparisons in Litters A and B because of their small numbers and because they were from 4 to 6 months old when the experiment was undertaken. However, the fever-treated Female 11-16 (Litter A) gained 21.04 per cent of her weight, while the untreated control doe in this litter, No. 11-18, gained only 14.45 per cent in weight in the same period. The treated buck gained 9.82 per cent. In Litter B the irradiated buck, No. 11-15, gained 9.84 per cent, while his doe, No. 11-19, likewise treated, made a percentage gain in weight of 20.67 per cent. The untreated doe, No. 11-17, showed a gain of 21.4 per cent,

which is slightly more than that of her treated sister. The percentage of gain in weight of the rabbits in Litters C, D, E, and F was similar although that of the treated rabbits, with one exception, was greater. The variation in percentage of weight gained between the irradiated and non-irradiated rabbits was never more than 6 per cent, while the average gain was from 2 to 3 per cent. The treated buck, No. 11-98 (Litter F), failed by 0.66 per cent to obtain as great a gain in weight as did his brother, No. 11-99. It is evident that 5 fevers given per week had no greater effect on the rabbits than 2 fevers per week. Of course, individual variation as to type and conformation of the rabbits must be considered. However, at the time when the litters were divided it was our aim to apportion them as equally as possible. The average percentage of gain in weight being practically always in favor of the rabbits given repeated fevers leads us to believe that the fevers stimulated their metabolism.

The protocols fail to show that repeated short wave fevers interfered with either the fertility of the bucks and does or with the size of the resultant litters when compared to the control group. The observations of Moore (8) indicate that the elevation of the temperature of the testes is a dangerous procedure, but in these experiments we failed to see any evidence of sterility being produced by the repeated elevation of the body temperature from 41–42°C. Rectal temperatures were recorded in these experiments, but by the use of thermocouples we have shown that normally the temperatures of the testes are from 1.5–2.5°C. lower than that of the rectum. Therefore, it is apparent that at no time during the irradiation was the temperature sufficiently elevated to injure the testes. The average age at which females become sexually mature and conceive is from 6 to 8 months. Allowing 31 days for the period of gestation, the controls conceived from 142 to 209 days of age, while the fever-treated rabbits conceived from 153 to 225 days of age. Thus, the average kindling age of the fever-treated females was 14 days older than that of the controls. This apparent delayed conception may be explained upon the basis of the frequent handling which may have disturbed the sexual relations of the male and female. However, an attempt was made to handle the controls as much as the irradiated group. As previously stated, when the fever-treated rabbits were brought from the animal house to the laboratory, the control

litter mates were brought with them and their temperatures were taken when they reached the laboratory and just before they were returned to the animal house. Nevertheless, the temperature of the irradiated rabbits was observed at intervals from 15 to 30 minutes during the exposure. Thus, the treated group was handled more because of the increased frequency with which their rectal temperatures were taken. With the exception of the differences above mentioned, we are aware of no change of the environmental factors in the two groups other than the repeated elevation of the body temperature by irradiation in an electrostatic field of short radio waves.

The average number of young born to the five control does was seven, while the average number in the litters born to seven fever-treated females was five and one-half. However, the average weight of the young from the irradiated females was 41 gm., while that of the young from the control females was 36.5 gm. It has been our experience that the average birth weight of young rabbits is inversely proportional to the size of the litter; that is, the larger the litter the smaller the average birth weight of the young. No definite deductions can be made from these data concerning the sizes of the litters, but if the fevers stimulated the metabolism of the adult, as will be discussed later, a similar factor may have had an effect on the growth of the fetuses *in utero*.

Only one of the control does, No. 11-17 (Litter B), raised three of her seven young, while two of the fever-treated does, Nos. 11-88 and 11-89 (Litter D), raised their litters. Rabbit 11-88, which received 2 fevers per week, raised all of her young, and Rabbit 11-89, given 5 fevers per week, raised five of her six young. It is disappointing that more of the treated and control females did not raise their litters, but because this failure was prevalent in both groups it is certainly not significant. All of the rabbits in the second generation matured to healthy and fertile adults. The litter of five contained three males and two females. They were inbred, with the exception of the extra male, who was bred to a normal female. In all of these matings the third generation was normal.

As a rule the treated and control females kindled living young, but occasionally a litter was born during the night and when the first observation was made of the nest, one or more of the litter was found

dead. None of the young showed any evidence of gross abnormality. The does of both groups had an abundance of milk but would not nurse the litter. Often the mothers cast them from the nest and gave the young no care. Since this attitude of the mother was similar in both groups, it is evidently not due to the irradiation or resultant fevers.

Welch (9) studied the effect of keeping rabbits in an incubator at temperatures from 41.5–41.8°C. for 3 weeks. He observed a loss of weight, but called attention to the differences between his artificial fever and that produced in the course of an infectious disease. In the former, heat loss was decreased, while in the latter it is not definitely known what changes in the heat-regulating mechanism occur. Nevertheless, it is evident that in the course of an infectious disease with elevation of body temperature, there is an increased heat production with or without a decreased heat loss in the body. He further quotes Naunyn as having kept a rabbit alive 13 days with an average body temperature of 41.5°C. In 1923 Walker (10) reported that an elevation of body temperature of 2° or 3°C. by the use of diathermy was accompanied by a 10 to 15 per cent increase in metabolism. Dubois (11) determined that during the course of a fever coincident with most infectious diseases, metabolism behaves according to Van't Hoff's law. He observed a 13 per cent increase for every 1°C. rise in body temperature. However, McCann and Barr (12) found that the basal metabolic rate in tuberculosis was within normal limits. Nasset, Bishop, and Warren (13) showed that when the body temperature of dogs was elevated from 5–7°C. by diathermy, the blood sugar was depleted, the non-protein nitrogen was increased, while the CO<sub>2</sub> content of the blood was diminished. These findings indicate a markedly increased metabolic rate during such fevers. That the short radio waves used to elevate the temperature of the rabbits have no specific effect on metabolism, and that the resultant heat of such irradiation is responsible for the increased weight, is indicated by the work of Luce-Clausen (14). She observed that near infra-red radiation (720 to 1120μ) accompanied by no noticeable rise of rectal temperature stimulated the growth of rachitic rats.

Although we know from previous experience with the production of short wave fevers in rabbits that it is possible to cause a loss of weight



from elevating the body temperature too high and maintaining it too long, we attempted to prevent such injury from overheating in this experiment. We anticipated an increased gain in weight in these rabbits because we have noted a gain in weight in those patients subjected to high frequency fevers as a therapeutic measure. We believe that the increased percentage of gain in weight of the fever-treated rabbits must be explained upon the basis of a stimulation of metabolism, or to an increased oxidation brought about by the increased acceleration of all body functions during the febrile reaction. However, these factors may also have caused a greater intake of food. It must be remembered that the type of fever produced in our experiments is very similar to that produced naturally in pathologic physiology. The short radio waves produce an internal heating that is entirely different from the elevation of the body temperature by warm air or warm water baths resulting from heat conductors.

#### SUMMARY AND CONCLUSIONS

1. The repeated elevation of body temperatures of male and female rabbits to 41° and 42°C. from 2 to 5 times per week by short radio waves (30 m.), beginning from 29 to 171 days of age and extending through their first period of gestation, failed to injure their growth or to interfere with mating, fertilization, or the development of young *in utero*. Litter mates were kept for controls.

2. The rabbits exposed to the short wave fevers showed, in the majority of cases, a greater percentage gain in weight than did the control litter mates.

3. The kindling age of the treated group was, on an average, 2 weeks older than the non-treated group.

4. The fever-treated females averaged five and one-half young per litter with an average weight of 41 gm., while the untreated females averaged seven per litter with an average weight of 36.5 gm.

5. The repeated elevation of body temperature by short radio waves is a safe procedure when temperatures greater than those within physiological limits are not employed.

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## EXPLANATION OF PLATES

## PLATE 34

FIG. 1. Litter D. Photographed Dec. 16, 1931. Age 14 months. Nos. 11-87, 11-88, 11-89, and 11-90 repeatedly irradiated with short wave (30 m.) fevers from 46 days of age until termination of first pregnancy. No. 11-91, control, untreated litter mate.

## PLATE 35

FIG. 2. Litter E. Photographed Dec. 16, 1931. Age 14 months. Nos. 11-93 and 11-94 repeatedly irradiated with short wave (30 m.) fevers from 44 days of age until termination of first pregnancy. Nos. 11-95, 11-96, and 11-97, controls, untreated litter mates.





Fig. 1

(Clark & Clark: Effects of fever temperature. II)





FIG. 1

(Continued from page 55)





Fig. 2

(H. A. and E. H. C. by temperature, II)





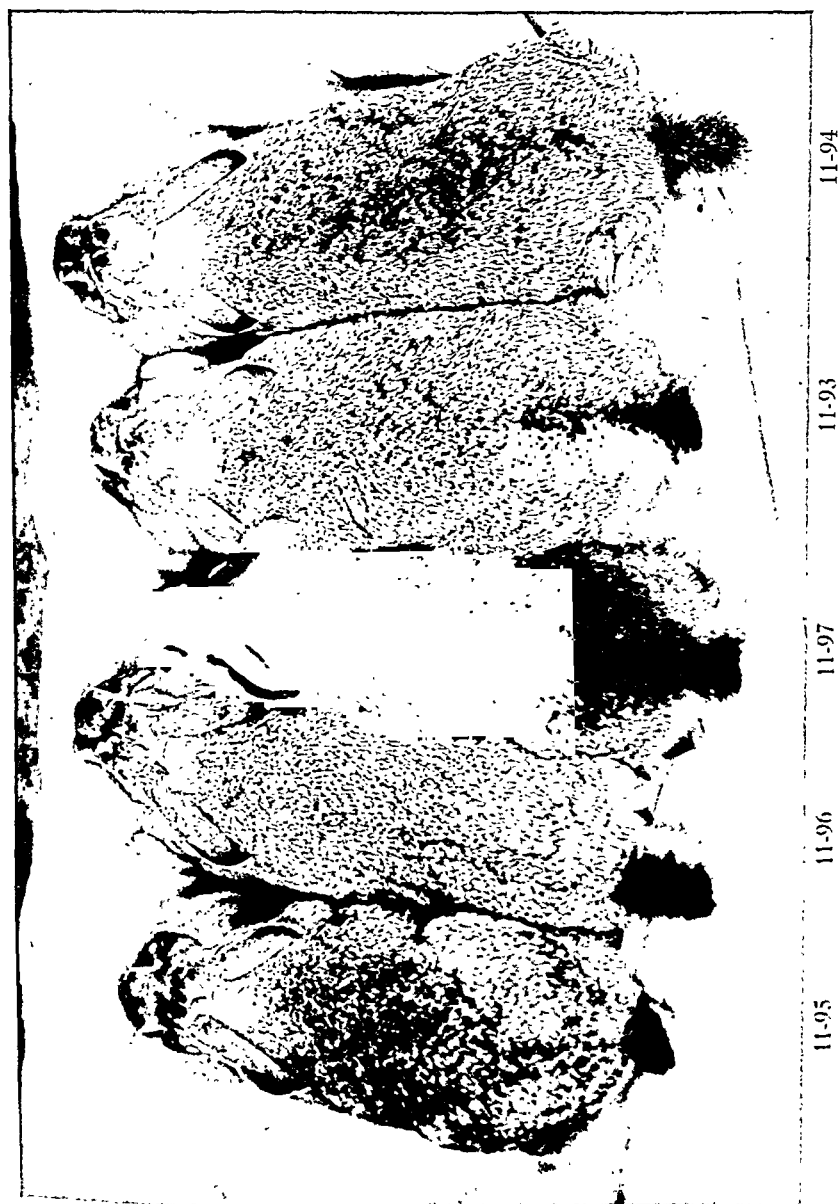


FIG. 2



# STUDIES ON THE PHYSIOLOGICAL EFFECTS OF FEVER TEMPERATURES

## III. THE THERMAL DEATH TIME OF *TREPONEMA PALLIDUM* IN VITRO WITH SPECIAL REFERENCE TO FEVER TEMPERATURES\*

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Wagner-Jauregg's (1) classical work on the treatment of dementia paralytica by the injection of *Plasmodium malariae* has stimulated much research on the therapeutic use of fever and its relation to infectious syndromes. The cause of remissions in paretics following fever therapy is not well understood, but evidence is being accumulated to show that the increased heat of the fever is an important and fundamental factor which has not been emphasized in the literature. We believe that fever temperatures are injurious to many of the pathogenic organisms, and are submitting evidence indicating that the spirochete of syphilis is especially susceptible to temperatures above normal. We realize that this is not a new idea, but it has been lost sight of, to a great extent, in the teaching of modern medicine as indicated by the use of antipyretics in febrile diseases.

The data which we present are the first of a series of studies on the thermal death time of various microorganisms. These studies offer a practical basis for establishing the duration of the fever period and the height of the temperature of patients subjected to artificial hyperthermia (2, 3). Although the results of our *in vitro* studies on the longevity of *Treponema pallidum* at fever temperatures are very sug-

\* This work was made possible by grants from the Rockefeller Foundation and the Research Laboratory of the General Electric Co.

gestive, a discussion of the clinical relations of the infection to the artificial therapeutic febrile reactions in the patient cannot be considered here. The clinical results of this method of treatment of paretics by others, as well as by us, have justified further investigation of fever as a therapeutic method of value in paresis. The data presented here suggest that the therapeutic fever may likewise be useful in the treatment of the earlier stages of syphilis.

Very little work has been reported on the thermal death time of *Treponema pallidum* *in vivo* or *in vitro*. This is no doubt due to the difficulty encountered in isolating and cultivating the organism, as well as to its failure to stain easily with dyes except under the complicated silver reduction technique. Another handicap is the uncertainty of animal inoculation in many instances and the long incubation period before the development of lesions. The majority of investigators consider that the thermal death time is short, and that because of its extreme parasitism it does not survive long in an environment slightly changed from its normal habitat.

### Method

We undertook the following experiments to determine the length of time *T. pallidum* could live *in vitro* at temperatures ranging from 37.5–42°C., 41.5°C. being approximately the maximum temperature that can be safely withstood by man for from 5 to 7 hours (3).

A total of 126 healthy, adult male rabbits, with large, well developed and completely descended testes were used in these experiments. Blood Wassermann tests were made on all rabbits before inoculation with the spirochete of syphilis. The Zinsser-Hopkins and the Nichols strains of *T. pallidum* were employed for this investigation. The description of a typical experiment follows:

Three rabbits were inoculated with the Zinsser-Hopkins strain of *T. pallidum*. After they developed either the typical syphiloma or chancre of experimental syphilis, under ether anesthesia the testes of these rabbits were removed aseptically, placed in a sterile mortar, and cut with sterile scissors into bits 3 to 4 mm. square. 30 cc. of a sterile physiological salt solution were then added and the tissues were ground to a fairly fine suspension. Dark-field examinations showed from 5 to 15 actively motile spirochetes in each microscopic field. With a 30 cc. glass Luer syringe and a long needle, amounts of 1 cc. of the extract were introduced into sterile glass vials of approximately 1.5 cc. capacity. The vials were sealed, labelled, attached to dental film holders, and immersed in water baths at temperatures of

37.5°, 39°, 40°, 41°, 41.5°, and 42°C. The variation of the temperature in these baths is not greater than 0.002°C. (A description of the baths is given in an accompanying paper.) Two vials were left on a laboratory table at room temperature (approximately 25°C.). At hourly intervals a vial was removed from each water bath, the seal broken, and the contents removed by a hypodermic syringe. 0.5 cc. of the extract was then injected into each testis of a rabbit, one rabbit being used for the contents of each vial. The contents of one of the vials kept at room temperature was similarly injected into rabbits after the 1st and 6th hours.

Two similar experiments were completed on the Nichols strain of *T. pallidum*. However, in the first of these an extract was prepared from a single rabbit and subjected to only one temperature each day. This was necessary because at that time only one water bath was available. It was feared that the variation in the number of spirochetes in extracts prepared from different rabbits might introduce a serious error. We found that this was not a factor, inasmuch as a thermal death time was obtained which was similar to that observed when a mixed extract was subjected to the different temperatures simultaneously on the same day.

Thirty-four rabbits were used for tests with each strain. Examinations were made at hourly intervals of the six different temperatures above mentioned, with the exception of 42°C. in which case injections were made for the first 4 hours only. Each rabbit was kept in an individual cage. The rabbits were inspected weekly for 14 weeks, after which time a second blood Wassermann examination was made.

When the development of lesions occurred in the testes, dark-field examinations were made, and in every case typical *T. pallidum* was found in varying numbers. Rabbits showing no lesions at the end of 3 months were considered negative. This was confirmed by the outcome of the Wassermann reactions and by further animal inoculation. These were conducted in one of the two following ways: Extracts were prepared from the testes and popliteal nodes removed aseptically under ether anesthesia from some of the group, and these were injected into the testes of other adult rabbits. The animals were observed 3 months for evidence of syphilis. The remaining animals of the group were given an intratesticular injection with a known virulent strain of *T. pallidum*. Several investigators (4-6) have stated that rabbits infected with syphilis will not develop typical lesions on reinoculation. We have used this criterion as an indication of the absence of syphilis in these rabbits. The rabbits which developed no lesions and in which the outcome of the Wassermann test remained unaltered have developed typical extensive syphilomata in the usual time on reinoculation. On the other hand, rabbits with healed and quiescent lesions have not developed syphilomata on reinoculation.

## RESULTS

The results of the examinations (as shown in Table I) are conclusive, and of great interest from several standpoints. The artificial conditions under which the spirochetes were kept apparently did not

injure them to any great extent, as determined by the injection of rabbits with the control extracts left standing in sealed vials at room temperature (25°C.) and in the water bath at 37.5°C. for 6 hours. In other experiments we have observed that even lower temperatures (22–23°C.) for 8 hours failed to injure the organism in such testicular extracts as evidenced by the fact that characteristic lesions of experimental syphilis were produced upon rabbit inoculation. There is some evidence (Duran-Reynals factor) (7) that a testicular extract is

TABLE I

*The Thermal Death Time of Two Strains of Treponema pallidum at Fever Temperatures*

Temperature °C.	Hrs. in water bath											
	Zinsser-Hopkins						Nichols					
	1	2	3	4	5	6	1	2	3	4	5	6
25.0	+					+						
37.5	+	+	+	+	+	+	+	+	+	+	+	+
39.0	+	+	+	—	—	+	+	+	+	+	—	—
40.0	+	—	—	—	—	—	+	+	—	—	—	—
41.0	—	—	—	—	—	—	+	—	—	—	—	—
41.5	—	—	—	—	—	—	—	—	—	—	—	—
42.0	—	—	—	—			—	—	—	—		

+ indicates infection in rabbit following intratesticular injection of heated extract.

— indicates no infection in rabbit following intratesticular injection of heated extract.

a most favorable medium for suspending the spirochetes and for their injections, because of its increasing the susceptibility of the host.

The hydrogen ion concentration of the extracts during the time they were exposed to the fever temperatures remained practically constant. A change of from only 0.1 to 0.5 pH, as determined by the electrometric method, was noted at the highest temperature for the maximum heating period. The slight change observed was toward the acid side.

Any criticism of the technique can be met by the fact that typical

*Results of Blood Wassermann Tests on Rabbits Injected Intratesticularly with  
Extracts Heated at Fever Temperatures*

Temperature of bath	Length of time in bath	Rabbit No.	Blood Wassermann before inoculation			Blood Wassermann 3 mos. after inoculation			Dark-field exami- nation	Lesions in testes
			Non-choles- terin- ized	Cholester- inized	Kahn	Non-cho- lesterinized	Cholester- inized	Kahn		
°C.	hrs.									
37.5 Control	1	16-27	—	—	—	++++	++++	++++	+	Chancres
	2	16-28	—	—	—	++++	++++	++++	+	Induration
	3									
	4	16-29	++	++	+++	++++	++++	++++	+	Chancres
	5									
	6	16-30	—	++	+++	+	++++	++++	+	Nodules
39.0	1	16-00	—	—	—	++++	++++	++++	+	Chancres
	2	16-05	—	—	—	++++	++++	++++	+	Nodules
	3	16-10	+	+++	+	++++	++++	++++	+	Nodules
	4	16-15	—	—	—	—	—	—	—	None
	5	16-19	+++	++++	++	+++	++++	++	—	None
	6	16-23	—	—	—	—	—	—	—	None
40.0	1	16-01	+	+	—	+	+++	+++	+	Induration
	2	16-06	—	—	—	—	—	—	—	None
	3	16-11	—	—	—	—	—	—	—	None
	4	16-16	—	++	—	—	—	—	—	None
	5	16-20	—	++	++	+-	+++	++	—	None
	6	16-24	—	+	—	—	—	+	—	None
41.0	1	16-02	—	++	+-	—	—	—	—	None
	2	16-07	—	+	++	—	+++	—	—	None
	3	16-12	—	—	—	—	—	—	—	None
	4	16-17	—	—	—	—	—	—	—	None
	5	16-21	—	—	—	—	—	+	—	None
	6	16-25	—	—	—	—	—	—	—	None
41.5	1	16-03	+	++	+++	—	++++	—	—	None
	2	16-08	+	+	—	—	—	—	—	None
	3	16-13	—	—	—	—	—	—	—	None
	4	16-18	—	—	—	+-	+-	—	—	None
	5	16-22	—	—	—	—	—	—	—	None
	6	16-26	—	++	++	++	++	+	—	None
42.0	1	16-04	+-	++	—	—	+-	—	—	None
	2	16-09	ac	ac	++	—	+-	+++	—	None
	3	16-14	+	++	++	+	++	++	—	None
	4	16-31	—	—	+	—	+	—	—	None
	5									
	6									

ac indicates anticomplementary.



experimental syphilis was produced by the injection of the control extracts maintained at 25° and 37.5°C. for 6 hours. It seems evident that the increased temperature was the fundamental factor in injuring or destroying the spirochetes. That this organism is so susceptible to slight changes in the temperature of its environment is remarkable.

The results of the blood Wassermann tests before and 3 months after inoculation on the series of rabbits injected with the Zinsser-Hopkins strain are given in Table II. In each instance the results with the non-cholesterinized and cholesterinized antigens are given, as well as those of the Kahn test. In several instances the blood of the uninjected rabbit showed some reaction that may be considered as non-specific or due to an infection with *Treponema cuniculi*. However, in no case was there a four plus reaction before injection with the three antigens used (see protocols). Our experience indicates that when typical syphilomas or chancres develop in rabbits' testes, the serum always shows a four plus reaction. We have noted no marked change in the Wassermann reaction during a 3 month period if infection was not established after injection. The results of our clinical findings and blood tests have been very consistent. Thomsen and Christensen (8) have reported that after rabbits were injected with *T. pallidum* extracts, a markedly positive Wassermann reaction developed regularly after from 5 to 6 weeks and continued for approximately 6 months, when it gradually disappeared regardless of the clinical course of the disease.

#### DISCUSSION

There are two general approaches to a study of fever temperature effects on *T. pallidum*: first, a consideration of the optimum temperature for its isolation and cultivation; and second, the resistance of the microorganism to temperatures higher than that normally observed in man.

The literature contains only a few reports on the optimum temperature for the isolation and cultivation of *T. pallidum*. It should be emphasized that some of the reported isolations of *T. pallidum* were evidently not those of the spirochete of syphilis, especially since these cultures failed to produce lesions when injected into experimental animals. In some of the standard texts on bacteriology a tem-

perature of 33.5°C. (Zinsser<sup>1</sup>) is stated to be most satisfactory for obtaining growths of this organism, while in others 37°C. is given as the optimum temperature. A survey of the literature of those men who have reported studies on the cultivation of *T. pallidum* shows that the majority used a temperature of 37°C. for their work. Schereschewsky (9) used a temperature of 37°C. for his isolation and cultivation experiments. He states that *T. pallidum* will grow at 40°C. Ungermann (10) has reported extensive investigations on the cultivation of spirochetes, and has studied the effects of temperature on their growth. He grew *Leptospira icterohaemorrhagiae*, *Spironema recurrentis*, *Spironema gallinarum*, and *T. pallidum* at temperatures of 30° and 37°C. All of the above organisms grew well at both temperatures. At 30°C. the spirochetes lived longer and *Spironema gallinarum* multiplied as rapidly as at 37°C. The spirochete of Weil's disease showed no growth at 25°C., a slight growth at 28°C., but the best results were obtained when the fresh cultures were grown at 37°C. for a few days and later placed at 25°C. *Spironema recurrentis* grew somewhat more slowly at 30° than at 37°C. but lived longer at the lower temperature. Ungermann presented fewer data on *T. pallidum*, but he states that as good growths were obtained at 30° as at 37°C. and that the spirochete of syphilis lived longer at the lower temperature. The similar experience of Inada, Ido, Hoki, Kaneko, and Ito (11) and of Noguchi (12) with the cultivation of *Leptospira icterohaemorrhagiae* of Weil's disease is likewise of interest. They demonstrated that the spirochete of Weil's disease grows between temperatures of 10° and 37°C., that it lives longer at temperatures from 25–30°C., and grows more slowly than at 37°C. Noguchi failed to get growth at 42°C. Although we realize that microorganisms from several genera in one family may vary considerably in their biological activities, it is obvious that these species of spirochetes pathogenic for man may be expected to have a similar optimum temperature for artificial cultivation. With the exception of the above citations, we have been unable to find any work to support the idea that a temperature lower than normal human body temperature is best for the isolation of this spirochete.

Few investigations of the resistance of *T. pallidum* to fever temperatures or to those above 45°C. have been made. In 1912 Arnheim (23) reported that spirochetes which he believed to be *T. pallidum* were not killed by incubation for several days at 45°C., nor by heating at 56°C. for 10 minutes. Nevertheless, all of his pathogenicity tests were negative. Bronfenbrenner and Noguchi (24) placed pure cultures of *T. pallidum* in physiological salt solution and exposed them to water bath temperatures of 37°, 40°, and 45°C. Control tubes were left at room temperature. After exposure for various lengths of time, sterile rabbit tissue and

<sup>1</sup> Zinsser has stated in a personal communication that he believes the use of a temperature of 33.5°C. for the isolation of *T. pallidum* evolved from observations that lesions of experimental syphilis developed best in the rabbit's testes, where the temperature is usually at that level.

ascitic agar were added to each tube, incubated at 37°C. for 10 days, and examined. They observed that their controls were viable for 12 hours at room temperature. The other cultures were viable for 6 hours at 37°, 1 hour at 40°, and 7 minutes at 45°C. No growth occurred after 10 minutes exposure at 45°C. They state that 37°C. for 6 hours injured the spirochetes because much less growth occurred in the tubes exposed at this temperature than in those at room temperature. However, they say that the organism would survive many hours at 45°C. if kept under strictly anaerobic conditions with properly balanced saline constituents and other nutrient substances. Zinsser and Hopkins (25) determined the resistance of cultures of *T. pallidum* mixed with cocci and bacilli, and placed on bits of cloth in diffuse light at room temperature varying from 21.5–25°C. The spirochetes were viable 11½ hours under these conditions, as were their controls in tubes in the same environment. When the same mixture of spirochetes and other microorganisms was placed on glass slides and allowed to dry under the above conditions, *T. pallidum* was dead after 1½ hours. Schamberg and Rule (26) heated an extract made from a rabbit's testis for 1 hour at 40°C. and found that it failed to infect on inoculation. They state that the thermal death time outside the body is 41°C. for 6 hours. Bessemans, Vergoullie, and Hacquaert (27) have been quoted on the effects of hot baths on human chancres. They observed that the healing of local lesions in rabbits and man occurred rapidly when exposed for 1 or 2 hours from 40–42°C. They conclude that exposure for 2 hours at 40°C., or for 1 hour at 42°C., will kill the spirochete of syphilis but will not injure the tissue cells. That *T. pallidum* has the same thermal death time *in vivo* as *in vitro* is much more difficult to determine. Wakerlin (28) has recently reported a case of syphilis due to an accidental laboratory infection with the Nichols (29) strain of *T. pallidum*. Such evidence indicates that this strain of spirochete has not lost its pathogenicity for man during many years of animal passage.

These observations suggest that the thermal death time of human strains is probably not longer than that of the strains of experimental syphilis used by us in these experiments. This will be discussed in another paper of this series.

#### SUMMARY AND CONCLUSIONS

1. The thermal death time of *Treponema pallidum* in extracts from lesions in rabbits' testes was determined *in vitro* at fever temperatures using the Zinsser-Hopkins and Nichols strains.

2. The criteria to determine the persistence of infectivity of the heated extract were the following: the development of lesions on inoculation into rabbits, dark-field examination of tissue from the lesions, the outcome of blood Wassermann tests, and of reinoculation tests.

3. The thermal death time of the two strains of spirochetes was approximately the same, although the Nichols strain was somewhat the more resistant. In the case of the latter 5 hours at 39°C., 3 hours at 40°C., 2 hours at 41°C., and 1 hour at 41.5°C., were required to render infective extracts innocuous to other rabbits.

4. The thermal death time of *T. pallidum* in testicular extracts *in vitro* at fever temperatures is so short as to suggest that induced fever may be useful therapeutically in human syphilis.

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# STUDIES ON THE PHYSIOLOGICAL EFFECTS OF FEVER TEMPERATURES

## IV. THE HEALING OF EXPERIMENTAL SYPHILIS LESIONS IN RABBITS BY SHORT WAVE FEVERS\*

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Wagner-Jauregg (1) was able to obtain clinical remissions in patients with dementia paralytica following a fever due to the injection of foreign proteins or to the tertian form of malaria. Believing that his results were produced chiefly by the effect of fever temperatures upon the spirochete of syphilis, we have studied experimentally the effect of fever on the healing of syphilitic lesions in rabbits. This can be done by placing the animal in a high frequency electrostatic field with the result that the temperature of the animal can be elevated and controlled at will, without introducing into the body any foreign material.

Since Bertarelli's (2) observation in 1906 that he could successfully produce syphilis in rabbits by the injection of spirochete-containing tissue from man, many studies on the treatment of experimental syphilis have been reported. A few workers have tried to influence the course of experimental syphilis in rabbits by the use of various methods to elevate the temperature of the animal. Weichbrodt and Jahnel (3) reported the healing of scrotal chancres in rabbits by placing the animals in an incubator at 41.0°C. for 1/2 hour twice daily over a period of from 3 to 5 weeks. Schamberg and Rule (4) were able to prevent the development of scrotal chancres by daily raising the body temperature of rabbits 2°C. with a total of eleven immersions in a hot water bath. The first bath was given on the 4th day after intratesticular injection of the spirochetes. Later, they (5) reported

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that they had cured two rabbits with scrotal chancres by submersing them on 15 successive days for 15 minutes in hot water baths at 45°C., which elevated their body temperature 5.2°F. and 5.8°F. (about 3°C.) respectively. Sublingual temperatures were recorded in their experiments.

In a previous report (6) we showed that short, repeated fevers prevented the development of syphilis in twenty-one of a series of twenty-five rabbits, when the treatments were begun 3 to 7 days after the intratesticular injection of extracts containing *Treponema pallidum*. In the absence of any obvious specific effects, we believe the elevation of temperature obtained when rabbits in the high frequency field produced by an oscillator (10,000 kilocycles) are exposed is due to heat from induced alternating currents and from the increased rate of vibration of the molecules of the cells produced by their alternate attraction to each of the plates in turn. As an accompanying paper shows, this type of fever is safe within the limits of the rabbit's body tolerance (42.5°C.).

The following experiments represent a continuation of the study of the effect of short multiple fevers on experimental syphilis in rabbits. Also, an attempt has been made to find the shortest number of treatments at a temperature of from 41–42°C. necessary to cure the animal of its infection. We have been able to demonstrate the efficacy of a prolonged single fever (41.5°C.). The influence of room temperature conditions (25–30°C.) on the testes must be guarded against when treating syphilitic lesions in the testes.

### Method

A group of thirty-seven adult male rabbits with large, well developed testes were used for these experiments. No pure bred animals were used and the rabbits varied considerably, resembling the Belgian hare, the New Zealand Red, the Flemish Giant, and the Albino, respectively. Seven were inoculated with the Zinsser-Hopkins strain of *T. pallidum* and the remainder with the Nichols strain. The following technique always produced good syphilomata or chancres.

A rabbit with well developed chancres in each testis was chosen from a previously inoculated series. Under ether anesthesia the testes and popliteal lymph nodes were removed aseptically, cut up into small bits in a sterile mortar, and ground with 10 cc. of a sterile physiological salt solution. A dark-field microscopical examination of the extract revealed from 3 to 25 active spirochetes per field. From 0.1 to 0.5 cc. of this suspension was injected into each testis of the group to be studied. The rabbits were kept in individual cages and inspected at weekly intervals until the chancres or syphilomata were well developed.

The rabbits were then divided into two groups. Sixteen were kept as controls to study the length of time required for spontaneous healing of the chancres, as well as the duration of the infectivity of *T. pallidum* in the rabbit after inoculation. From 30 to 395 days after injection, the sixteen control rabbits were sacrificed and extracts made from their testes and popliteal lymph nodes, as previously described. The suspensions were then injected intratesticularly into an equal number of normal, male, adult rabbits, with well descended testes (see Table I). This reinoculated group of rabbits were then inspected at weekly intervals for evidence of the development of syphilis as indicated by gross lesions, blood Wassermanns, and dark-field examination.

The other group of twenty-one inoculated rabbits were subdivided and heated by irradiation in the short wave electrostatic field (Tables II and III). The fever treatments were started at various stages of development and healing of the testicular lesions. The number and length of the heating periods, as well as the period between the end of the treatment and reinoculation, were varied in order to study the effects of these different time intervals.

The heating was accomplished very simply by exposing the rabbits to high frequency waves (10,000 kilocycles) in a field between two aluminum plates of a high frequency oscillator (6). The animals were placed in an orange crate or a glass battery jar, and the field strength regulated to elevate the temperature of the rabbit within 20 to 30 minutes to about 41.5°C. The animals were removed from the field every 15 minutes for rectal temperature observations after which they were returned to the container and the heating continued if their temperature was to be elevated further. When it was to be maintained at a given level, the field strength was reduced accordingly or the current turned on and off at the proper intervals. After the treatment was completed the animal was placed on the cool cement floor or in a box while defervescence occurred. The period of cooling varied considerably with the room temperature and with the type of rabbit. Those with thick fur, such as the Angora rabbits, required an hour or more to return to the temperature which they had prior to the irradiation.

The first fourteen animals were heated from 41–42°, 5 or 6 times each week until the scrotal chancres had completely healed and the scabs had fallen off. This usually required 3 weeks, although in some instances 4 weeks of treatments were necessary. These daily treatments were used to imitate the paroxysms of malaria fever since this type of febrile reaction is used for the treatment of paresis. Later, rabbits were given longer and fewer treatments in order to determine the minimum number of fevers at the above temperatures necessary to kill the spirochete. As noted in Table III, only one 6 hour treatment was given to rabbits with well developed syphilis and found to be effective.

When the designated number of fevers were completed, the rabbits were sacrificed at intervals up to 214 days. Extracts of their testes and popliteal lymph nodes were injected into an equal number of normal, male, adult rabbits according to the procedure already described for the controls. Prior to injection, dark-field



examinations of the inoculated material were made in almost every case. Histological examinations were made on some of the tissues, but Levaditi stains for spirochetes and the usual hematoxylin and eosin stain were of little value in proving that the infection had been cured.

All of the rabbits irradiated possessed well developed syphilomata or chancres in the testes before treatment except No. 12-12. Clinically, the lesions in this case had healed spontaneously, but the animal was no doubt still infectious as shown by the findings in our control series. Rabbit 4-97 was overheated. His rectal temperature registered 46.6°C. during a treatment. Upon this finding, the testes and popliteal lymph nodes were immediately removed under ether anesthesia, an extract was prepared and injected into another rabbit.

### RESULT

It will be noted from Table I that the inoculation of a group of normal male rabbits with extracts of testes and popliteal lymph nodes from the controls gave positive results in every instance. In some of the control rabbits, the extract from the testes was injected into one testis, while the extract from the popliteal lymph nodes was injected into the other testis. However, in most cases a bilateral intratesticular injection of 0.5 cc. of a single extract prepared from the testes and lymph nodes was made. Our experience has shown that in several cases extracts from the popliteal lymph nodes failed to infect, while the extract from the testes gave positive results. In no instance have we found evidence of *T. pallidum* by animal inoculation in the popliteal lymph nodes when the testicular extract failed to infect.

The finding that *T. pallidum* remained in the popliteal lymph nodes and in the testes for a long time after spontaneous clinical healing of the lesions confirms that of other observers. In the group of controls, viable spirochetes were found 395 days after injection. Good lesions developed in the reinoculated series after this comparatively long interval. No clinical evidence of generalized lesions or of extension of the infection beyond the testes, inguinal, and popliteal nodes occurred in the control group of rabbits. The average time required for the development and clinical healing of the chancres in the untreated rabbits was 4 months. Occasionally a small chancre would heal in 3 months when only some thickening of the scrotum, or perhaps small nodules in the epididymis or testis, remained. However, this material was still infectious.

The results of the reinjections of extracts from the testes and popliteal lymph nodes of the treated group are tabulated in Tables II and III. It will be noted that with one exception (Rabbit 4-99), negative reinoculation results were obtained with the various fevers and intervals of irradiation used. We continued the fever of the malarial type (*i.e.* multiple short periods) until the chancre had completely

TABLE I  
*Control Rabbits*

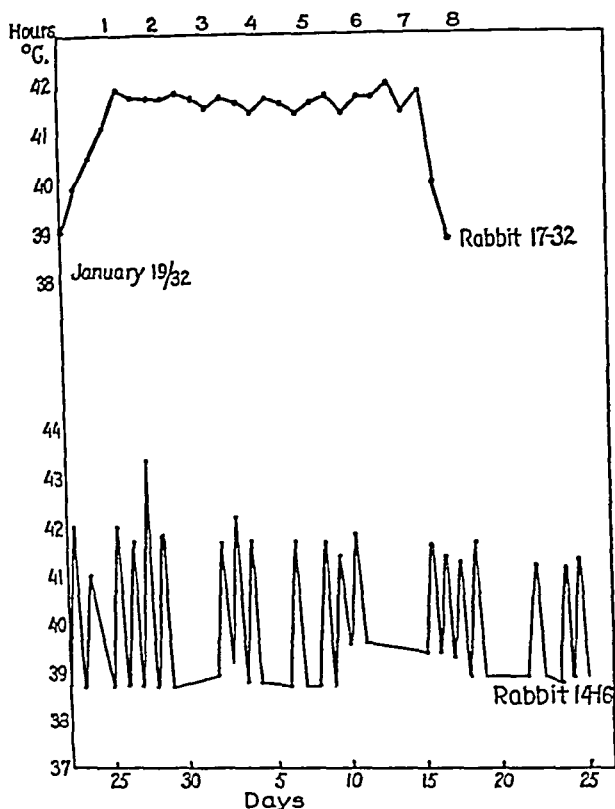
Rabbit No.	Date of injection	Strain	Amount injected	Interval between 1st injection and reinoculation	Dark-field examination	Date of reinoculation	No. of rabbit injected	Result of reinoculation
			<i>cc.</i>	<i>days</i>				
3-74	Sept. 18, 1929	Nichols	0.1	30	+	Nov. 18, 1929	4-53	Positive
13-41	Feb. 5, 1931	Nichols	0.5	43	+	Mar. 20, 1931	13-86	Positive
7-39	Apr. 1, 1930	Nichols	0.2	86	+	June 26, 1930	9-84	Positive
14-26	May 15, 1931	Zinsser-Hopkins	0.5	87	+	Aug. 11, 1931	15-87	Positive
16-29	Oct. 13, 1931	Zinsser-Hopkins	0.5	93	+	Jan. 14, 1931	17-31	Positive
7-34	Apr. 1, 1930	Nichols	0.2	115	+	July 25, 1930	10-57	Positive
13-40	Feb. 5, 1931	Nichols	0.5	123	+	June 8, 1931	14-58	Positive
13-86	Mar. 20, 1931	Nichols	0.5	131	+	July 29, 1931	15-80	Positive
13-71	Mar. 7, 1931	Nichols	0.5	144	+	July 29, 1931	15-78	Positive
13-76	Mar. 7, 1931	Nichols	0.5	157	S	Aug. 11, 1931	15-84	Positive
13-75	Mar. 7, 1931	Nichols	0.5	172	—	Aug. 26, 1931	15-91	Positive
4-96	Jan. 14, 1930	Nichols	0.1	175	—	July 8, 1930	10-15	Positive
3-89	Sept. 18, 1929	Nichols	0.1	266	—	June 11, 1930	9-66	Positive
7-32	Apr. 1, 1930	Nichols	0.2	321	—	Feb. 16, 1931	13-51	Positive
2-51	July 22, 1929	Nichols	0.2	353	—	July 10, 1930	10-17	Positive
3-88	Sept. 18, 1929	Nichols	0.1	395	—	Nov. 18, 1930	4-54	Positive

healed. As a rule, an average of 20 fevers was required, although frequently more in the first experiments. This was found to be due to the very rapid cooling of the infected tissues and especially the testes, when the animals were removed from the oscillator and placed on a table at room temperature in order to obtain the rectal temperature. We (6) have previously demonstrated by the use of a thermocouple that the temperature of the testes is from 1-2°C. lower than

TABLE II  
*Irradiated Rabbits*  
*Multiple Short Fevers*

Rabbit No.	Date of injection	Strain	Amount injected in each testis	Interval between injection and beginning of treatment	Lesions at beginning of treatment	Dark-field examination	No. of treatments	Duration of treatment	Maximum rectal temperature	Interval between beginning and end of treatment	Interval between end of treatment and reinoculation	Dark-field examination	Date of reinoculation	No. of rabbit injected	Result of reinoculation
3-73	Sept. 18, 1929	Nichols	0.1	61	Chancre	+	18	17	41.9	36	214	-	July 21, 1930	10-44	Negative
3-90	Sept. 18, 1929	Nichols	0.1	85	Chancre	+	24	33½	42.0	60	171	-	July 31, 1930	10-72	Negative
4-97	Jan. 14, 1930	Nichols	0.1	41	Chancre	+	16	19	46.6	35	0	-	Mar. 31, 1930	7-25	Negative
4-99	Jan. 14, 1930	Nichols	0.1	93	Chancre	+	55	84	42.6	67	15	+	July 8, 1930	10-16	Positive
7-31	Apr. 1, 1930	Nichols	0.2	62	Chancre	+	34	43½	42.8	79	8	-	July 29, 1930	10-69	Negative
7-33	Apr. 1, 1930	Nichols	0.2	66	Chancre	+	30	36½	42.2	45	10	-	July 31, 1930	10-71	Negative
7-36	Apr. 1, 1930	Nichols	0.2	62	Chancre	+	37	47½	42.0	49	4	-	July 25, 1930	10-58	Negative
7-38	Apr. 1, 1930	Nichols	0.2	62	Chancre	+	25	34½	42.2	31	27	-	July 30, 1930	10-70	Negative
13-37	Feb. 5, 1931	Nichols	0.5	67	Chancre	+	20	69½	42.8	32	24	-	June 8, 1931	14-61	Negative
13-38	Feb. 5, 1931	Nichols	0.5	67	Chancre	+	19	64½	43.2	32	26	-	June 10, 1931	14-64	Negative
13-73	Mar. 7, 1931	Nichols	0.5	88	Syphiloma	+	20	63½	42.6	38	18	-	July 29, 1931	15-79	Negative
13-74	Mar. 7, 1931	Nichols	0.5	88	Syphiloma	+	20	64½	42.7	38	4	-	July 13, 1931	15-44	Negative
13-78	Mar. 7, 1931	Nichols	0.5	76	Chancre	+	20	70½	43.3	34	11	-	July 5, 1931	14-82	Negative
14-16	Mar. 20, 1931	Nichols	0.5	63	Chancre	+	20	66	43.3	34	12	-	July 6, 1931	14-83	Negative

that of the rectum. Recently using a similar method we studied the rapidity of defervescence in various parts of the body after removal of the rabbit from the short wave field. An example of the difference in the rates of cooling of the rectum and testes follows: In 25 minutes after discontinuing irradiation and removal to room temperature the



GRAPH 1. Types of fever produced in rabbits. Upper curve, one continuous fever of 6 hours. Lower curve, series of short unsustained fevers.

rectal temperature dropped  $0.8^{\circ}\text{C}.$ , while that of the testes fell  $3.0^{\circ}\text{C}.$  It was likewise observed that the temperature of a chancre is lower by at least  $1.0^{\circ}\text{C}.$  than that of a syphiloma embedded in the substance of the testes. The temperature gradients will be discussed in detail in another paper of this series. Therefore, the temperature in the syphilitic lesions remained at the high level of the rectal temperature

TABLE III  
*Irradiated Rabbits*  
*Prolonged Fevers*

Rabbit No.	Date of injection	Strain	Amount injected in each testis	Interval between injection and beginning of treatment	Lesion at beginning of treatment	Dark-field examination before treatment	No. of treatments	Duration of treatment	Maximum rectal temperature	Interval between beginning and end of treatment	Interval between end of treatment and reinoculation	Dark-field examination after treatment	Date of reinoculation	No. of rabbit injected	Result of reinoculation	Duration of observation
14-27	May 15, 1931	Zinsser-Hopkins	0.5	90	Syphiloma	+	3	18½	41.9	18	9	—	Sept. 9, 1931	15-94	Negative	120
14-59	June 8, 1931	Zinsser-Hopkins	0.5	67	Syphiloma	+	2	12	41.9	9	4	—	Oct. 26, 1931	15-90	Negative	120
12-12	Dec. 9, 1930	Nichols	0.5	116	Syphiloma	+	1	9½	43.7	1	1	—	Apr. 8, 1930	14-20	Negative	120
16-01	Oct. 13, 1931	Zinsser-Hopkins	0.5	98	Syphiloma	+	1	6	41.7	0	3	—	Jan. 22, 1932	17-57	Negative	120
16-05	Oct. 13, 1931	Zinsser-Hopkins	0.5	98	Syphiloma	+	1	6	41.8	0	3	—	Jan. 22, 1932	17-55	Negative	120
16-28	Oct. 13, 1931	Zinsser-Hopkins	0.5	98	Syphiloma	+	1	6	43.0	0	3	—	Jan. 22, 1932	17-58	Negative	120
16-32	Oct. 13, 1931	Zinsser-Hopkins	0.5	98	Chancre	+	1	6	41.9	0	3	—	Jan. 22, 1932	17-56	Negative	120

only while the animals were in the oscillator, which, as a rule, was not more than 2 hours in the case of the short multiple exposures. These findings may explain the failure to kill the spirochetes in Rabbit 4-99, despite the large number (55) of short treatments. This rabbit had very extensive chancres on both scrotal walls, practically the entire scrotum being involved. These chancres healed very slowly and much scar tissue remained after healing. It was difficult, for some unknown reason, to establish a fever in this rabbit and in many treatments the rectal temperature did not reach more than 40.5°C. In later experiments this condition was rectified by treating the animals in a room with a temperature of from 35-37°C.

#### DISCUSSION

The important observation in the treated series (Table III) is the fact that a fever at 41-41.5°C., sustained for 6 hours was as effective in injuring or destroying *T. pallidum* in the rabbit as was a series of from 20 to 30 short pyrexias. In the case of those animals exposed to short, repeated fevers, there was a total maximum of from 17 to 70 hours in the high frequency field during which time their body temperature was elevated above normal. It is interesting to find that one treatment of 6 hours in the field is just as effective as the greater number of hours of irradiation from the many treatments. This was true, regardless of the size or type of lesion in the testes. Of course, complete healing of the syphilitic lesion could not occur in the short interval of from 3 to 9 days after one sustained fever treatment, at which time the rabbit was sacrificed and extracts from the testes and lymph nodes reinjected. Nevertheless, a complete change in the character of the lesion was noted in this period. The syphilomas in the testes became softer and less extensive and the same was true of the indurated testes. The chancres showed evidence of rapid healing, were dry, decreased in size, became markedly umbilicated, while the periphery of the scabs was elevated and free from the tissue healing beneath them.

We have included in Tables II and III the maximum temperature reached during the fever treatment, which in some cases was higher than we desired to produce. Although rectal temperatures were taken every 15 minutes, it was difficult to avoid a sudden rapid eleva-

tion of temperature, especially on summer days when the treating room was warm and had a high humidity. Because of the lower temperature level of the testes, it is evident that at no time, with the exception of Rabbit 4-97 that was killed by overheating, was the temperature of the testes above that of 41.5°, and in most instances for only a brief interval at this point.

The dark-field examinations of extracts from the site of the lesions, or the testes after the lesions had disappeared in the case of the fever-treated group, were always negative. Occasionally suspicious bodies were observed, but we could not in any case see definite typical motile spirochetes. The microscopic bodies seen may have been dead spirochetes. The histological sections of those lesions observed after staining with Levaditi's method failed to reveal any spirochetes, although this is not as good evidence as the reinoculation test (see tables).

The results are in accord with our earlier studies (6), in which we prevented lesions of experimental syphilis from developing by producing in rabbits a series of about 20 short wave fevers, beginning from 3 to 7 days after intratesticular injection of *T. pallidum*. The data show that single fevers are just as effective in healing the lesions and in destroying the spirochete in the body of the host. It is evident from an accompanying paper (7) that the increased heat of the fever provides an unfavorable environment for the spirochetes that either destroys or injures them so that they lose their infectivity. We do not know whether in syphilis the elevated temperature also stimulates or activates those factors in the body that are concerned with its protection against infection. However, in studies on gonorrhea we have observed increased phagocytosis during artificially induced fever. This leads us to believe that such factors may play a prominent part in syphilis. Two of the rabbits, Nos. 3-73 and 3-90, were allowed to live 214 and 171 days, respectively, after treatment. Since their testicular and nodal extracts failed to produce syphilis on injection into other rabbits, it seems plain that the spirochetes must have been killed and not injured only temporarily.

The fertility of some of the rabbits was tested after treatment by breeding to normal females. Some of the males were sterile temporarily, while normal litters were obtained in other cases. One male

failed to cause conception in a female known to be fertile when bred to normal males. The extent of the injury caused by the lesions of syphilis no doubt had much to do with the production of sterility. We have noted in untreated syphilitic males that an extensive lesion causing severe injury to both testes resulted in sterility.

Some of the rabbits lost weight during the frequent heatings, but it was recovered rapidly after irradiation ceased. In general, we failed to see any injury to the general health of the rabbits, except in the case of Rabbit 4-97 which was overheated.

The thermolability of *T. pallidum* is again emphasized. The findings suggest the practicality of fever therapy in the treatment of acute as well as chronic syphilis in man.

#### SUMMARY AND CONCLUSION

1. Multiple, unsustained fevers (41–42°C.) produced by irradiation in a high frequency electrostatic field (10,000 kilocycles) destroyed *T. pallidum* in rabbits with active syphilitic lesions as determined by the injection into normal rabbits of extracts prepared from their testes and popliteal lymph nodes.

2. One febrile period of 6 hours at a temperature of 41.5–42°C. was likewise found to be sufficient to destroy *T. pallidum*.

3. Infection with *T. pallidum* persisted in a control series of untreated rabbits for as long as 395 days after inoculation, but clinical healing occurred in from 3 to 4 months after injection.

4. The time interval between inoculation and fever treatment, or between the end of the fever treatment and reinoculation, did not affect the results.

5. The fever treatment was effective at any stage of experimental syphilis in rabbits.

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# THE ETIOLOGY OF BARTONELLA MURIS ANEMIA OF THE ALBINO RAT

## THE ISOLATION OF BARTONELLA MURIS

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PLATE 36

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As the present paper will show, it has been possible to isolate an organism in pure culture from the blood of splenectomized rats suffering from *Bartonella muris* anemia. A severe anemia was produced by the injection of this organism into 3 week old rats, rabbits, guinea pigs and young mice, all with intact spleens. The organism when injected into adult splenectomized Wistar Institute rats of non-carrier stock produced a mild anemia. During the height of the anemia occasional *Bartonella* bodies were found on the red cells. The strain of *Bartonella muris* was recovered in pure culture from these animals. The blood of the 3 week old rats and 3 week old rabbits, when injected into other immature animals of the same species, produced a transmissible infectious anemia.

Noguchi (1) isolated two organisms from the blood of a splenectomized rat, both of which he believed resembled *Bartonella muris* in morphology. The first grew on leptospira medium, but did not grow on ordinary culture media. This was a diphtheroid and was non-pathogenic for normal rats. From the blood of the same animal another minute Gram-negative non-motile bacterium was isolated on a blood plate. This organism grew on blood agar, was hemolytic but did not produce acid in sugars. It caused an acute orchitis in normal rats when injected intratesticularly.

Battistini and Weiss (2) report the isolation of an organism of the *Salmonella* group from the blood of splenectomized wild rats of Lima. They mention no experimental data supporting their contention of an etiological relationship with *Bartonella muris* anemia. They stress the similarity of the human Oroya fever to *Bartonella muris* anemia of the rat, and report unsuccessful attempts to transmit the rat anemia to monkeys, mice or guinea pigs with whole blood injections of anemic rats.

Lwoff and Vaucel (3) injected the blood of a dog which had been infected with *T. cruzi* (from a rat) into mice with intact spleens. *Bartonella* bodies appeared on the red cells in the mice. The *Bartonella* infection was probably carried from the rat through the dog. The *Bartonella* infection was transferred from mouse to mouse by injections of whole blood for 28 passages. These investigators isolated an organism from one of these mice on Noguchi's leptospira medium at 22-37°C. in 14 days and in N. N. N. medium (Nicolle, Novy, McNeal) in 10 days. The bacteria were both motile and non-motile, retained motility for 10 days and resembled morphologically *Bartonella bacilliformis*. In a mouse injected with 6 drops of the culture *Bartonella* bodies were occasionally seen on the 5th and 7th days. 26 days later the mouse was injected with the blood of a mouse heavily infected with *Bartonella muris* and developed a severe infection. Two other mice were injected with the culture. One remained free of infection and the other showed occasional *Bartonella* bodies on the red cells on the 4th to 7th day. The strain was carried through 3 subcultures in artificial media. It produced only a feeble infection and afforded no protection against subsequent infection.

### Methods

The method of cultivation of *Bartonella muris* was the same as that employed by Noguchi for the isolation of *Bartonella bacilliformis*. The blood was withdrawn from the heart of an adult splenectomized rat at the height of the anemia in an equal volume of sterile isotonic sodium citrate solution. Dilutions of 1/10, 1/100, 1/1000, 1/10,000 and 1/100,000 were made of this blood with citrate. 0.4 cc. of each dilution was inoculated into tubes of Noguchi's leptospira medium at a pH of 7.4 and incubated at a temperature of 25°. Successful results were obtained in only two of many instances attempted. Growth appeared within 10 to 12 days as a fine cloud at the upper layer of the medium. By heavy seeding (0.2 cc.) the culture was transplanted to tubes of leptospira medium and after 2 transfers could be grown on blood agar slants.

### Morphology

In the original culture there were scattered bacilli and coccobacilli with bent forms. They are fine rods varying from 0.4 to 3.2 micra in length and 0.2 to 0.4 micron in width with a predominance of short forms. On solid media the bacillary forms predominate and tend to clump with occasional thread formation. The sides of the bacilli are straight and the ends rounded. They are Gram-negative and very actively motile<sup>1</sup> on both the solid and semisolid media. When re-transplanted from the solid to the semisolid medium the shorter pleo-

<sup>1</sup> After 4 months on artificial media the motility of the organisms markedly diminished.

morphic forms are predominant. After repeated subculture and animal passage the organisms are apt to be somewhat plumper than in the original cultures. Flagella are present.

### *Cultural Characteristics*

Before animal passage the organism grew only in the presence of rabbit, horse or human blood which was added to hormone agar. This original strain has been grown on artificial media for 3 months and is still under observation.

On blood agar minute colonies appear in 48 hours. These are at first barely visible, translucent and round. They gradually increase in size and in a few days coalesce, forming a thin filmy tenacious growth on the surface of the medium. The color of older cultures is grayish with a tinge of yellow. The blood in the medium is not hemolyzed. 10 per cent solutions of each of sixteen sugars were added to Hiss serum water containing 0.2 per cent hemoglobin solution. On the sugar media containing glucose, maltose, saccharose, mannite, lactose, mannose, xylose, arabinose, raffinose, galactose, dextrin, levulose, salicin, inosite, inulin or dulcitol, neither gas nor acid was produced during a period of 10 days. Litmus milk is slightly coagulated in 48 hours but no acid is formed. After animal passage the organism grew on glucose bouillon and ascitic agar without blood. In glucose bouillon the growth is limited to the upper layer. The organisms are actively motile on liquid, semisolid and solid media.

Cultures on solid media have a sweet odor, resembling canned pineapple. Old cultures, particularly on blood, have a faint herring odor. In liquid media after 10 days to 2 weeks a faint green pigmentation of the medium occurs. This pigment is insoluble in chloroform.

The optimal temperature for growth is 25°C., though slight growth occurs at 37°C. At room temperature the cultures in leptospira medium retain motility and viability for 38 days as determined by subculture.

*Bartonella muris* is differentiated from other Gram-negative motile bacilli by its cultural and biological characteristics.

### *Infection of 3 Week Old, 30 Gm. Rats with Bartonella muris Cultures*

It has been demonstrated by Ford (4) and confirmed by the authors (5) that 3 week old, 30 gm. rats and rabbits with intact spleen will develop an anemia if injected with the blood of an anemic splenectomized rat.

Fourteen 3 week old, 30 gm. rats received 0.5 cc. of a 48 hour growth on leptospira medium or washings from young cultures on blood agar slants, intraperi-

toneally. Within 24 hours the rats became severely anemic, the hemoglobin dropped to below 40 per cent and in many instances the red cells fell to below 2,500,000 per c.mm. The anemia in the rats infected with the original culture was not as severe as that in the rats infected with subsequent subcultures. In both cases the anemia continued from 3 to 5 days after which the animals recovered. *Bartonella* bodies were found occasionally on the red cells. They were never numerous. Blood cultures made 48 hours after the onset of the infection were positive for *Bartonella muris*. Occasionally cultures of the liver and spleen were

TABLE I

3 week old rats injected with 0.5 cc. culture of *Bartonella muris* (Strain I-4°). (Sample protocol.)

Rat	Date		Red cell count	Hb (Dare)	<i>Bartonella</i> bodies	Blood culture
	1932			per cent		
K <sub>1</sub>	Mar. 29	Before injection	5,500,000	110	Occasional <i>B. muris</i> bodies " "	Positive blood culture
	" 30	After injection	3,200,000	40		
	" 31	" "	3,000,000	38		
	Apr. 1	" "				
	" 3					
	" 4					
K <sub>2</sub>	Mar. 29	Before injection	4,900,000	92	Occasional <i>B. muris</i> bodies	
	" 30	After injection	2,600,000	45		
	" 31	" "	3,200,000	48		
	Apr. 1	" "	5,000,000	70		
	" 3	" "	4,800,000	75		
	" 4	" "	5,100,000	85		

positive. The organism was reisolated from the blood of the animals 1 to 5 days after injection. Colonies appeared within 48 hours after inoculation of the medium, at 25°C.

The culture reisolated from infected animals was pathogenic for other young rats in the same manner as the original culture.

The blood of the infected rats was infectious for other 30 gm. rats and the anemia produced was transmitted to other animals in series by injection of whole blood.



DIAGRAM 1

*Infection of Suckling Rabbits with Bartonella muris Cultures*

The susceptibility of the 3 week old rabbits with intact spleens (suckling) to *Bartonella muris* infection by the injection of whole blood of an anemic splenectomized rat as demonstrated by Ford (4) was likewise utilized to test the pathogenicity of the organism isolated from the anemic rat (*Bartonella muris*).

Three 3 week old rabbits received intravenously 0.5 and 1 cc. of a young culture of *Bartonella muris* on leptospira medium. A moderate anemia developed within 2 to 3 days, and reached its height on the 5th day after injection. The hemoglo-

TABLE II

3 week old rabbit injected with 1 cc. culture *Bartonella muris* (24 hour slant) intravenously. (Sample protocol.)

Rab- bit No.	Date		Red cell count	Hb (Dare)	<i>Bartonella</i> bodies	Blood culture
	1932			per cent		
4	Apr. 8	Before injection	5,800,000	80		Negative
	" 9	After injection	5,600,000	75		
	" 10	" "	4,500,000	68		
	" 11	" "	4,000,000	60	<i>B. muris</i> bodies (occasional)	
	" 12	" "	4,200,000	62		
	" 13	" "	3,300,000	60		Positive cul- ture
	" 14	" "	3,000,000	50	Occasional <i>B.</i> <i>muris</i>	
	" 16	" "	4,000,000	60		
	" 21	" "	7,000,000	100		

bin dropped to 50 per cent and the red cell count to 3,000,000 per c.mm. *Bartonella muris* bodies were occasionally seen. On the 5th day the blood culture was positive for *Bartonella muris* (see Table II).

Two adult rabbits injected repeatedly with large amounts of the culture intravenously showed no evidence of disease.

*Infection of 3 Week Old Guinea Pigs with Bartonella muris Cultures*

Three 3 week old guinea pigs and three adult guinea pigs were injected intraperitoneally with 0.5 cc. of a young culture of *Bartonella muris* on leptospira medium. The three young animals became severely anemic within 24 hours. The adult animals remained unaffected. The anemia continued for 3 to 5 days with recovery of the animals. The *Bartonella* bodies were found on the red cells,

very sparsely scattered.<sup>2</sup> The organism was recovered from the blood on the 2nd and 5th days of the anemia. In one instance (see Table III) the hemoglobin

TABLE III

3 week old guinea pig injected with 1 cc. culture *Bartonella muris*. (Sample protocol.)

Date		Red cell count	Hb (Dare)	<i>Bartonella</i> bodies	Blood culture
1932			per cent		
Mar. 29	Before injection	4,900,000	80		
" 29	Injected with 1 cc. culture				
" 30	After injection	2,500,000	48	Occasional <i>B. muris</i> body	
" 31	" "	2,200,000	25	" "	Positive culture <i>B. muris</i>
				Killed. Autopsy: fatty liver, watery blood, marked anemia, congested spleen	

TABLE IV

7 week old mouse injected with 0.3 cc. culture of *Bartonella muris*. (Sample protocol.)

Date		Red cell count	Hb (Dare)	<i>Bartonella</i> bodies	Blood culture
1932			per cent		
May 27	Before injection	5,500,000	120		
" 28	After injection	5,000,000	110		
" 29	" "	5,200,000	110		
" 30	" "	6,000,000	105		
" 31	" "	6,500,000	105	Occasional	
June 1	" "	3,100,000	40	"	
" 2	" "	2,700,000	29	"	Positive
		(Killed)			

dropped to 25 per cent and the count to 2,200,000 per c.mm. Autopsy of this animal (killed by heart puncture) revealed the pathological picture of *Bartonella*

<sup>2</sup> Rybinsky (6) observed bodies on the red blood cells of adult guinea pigs that were previously repeatedly injected with trypan blue, and of guinea pigs infected with *T. brucei*. He describes these as round and oval, staining a dark violet color with Giemsa and measuring 1 to 2 microns in size. He suggests the name of *Bartonella ukrainica* for this type of *Bartonella* infection.



TABLE V

*The Production of Bartonella muris Anemia in Adult Splenectomized Rats of Non-Carrier Stock (Wistar) Following Injections of Bartonella muris Cultures. Cultures Were Injected 2 Days after Splenectomy*

Injected with 5 cc. culture	Length of time after splene- ctomy	Blood counts													
		Days after injection													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	2 days	100% 7500T	95% 7500T	90% 6000T	90% 6700T	105% 7500T	100% 7000T	110% 7000T	110% 7800T	105% 7500T	100% 7200T	78% 6200T	78% 7500T	90% 6700T	90% 7100T
2	2	105% 7600T	90% 7300T	80%* 6300T	76%* 5900T	62%* 4500T	80% 4750T	100% 5800T	50% 4800T	50% 4000T	95% 4500T	105% 5800T	110% 7000T	105% 6500T	
3	2	99% 8000T	110% 8200T	100% 6900T	90% 7100T	90% 8000T	85% 7900T	105% 7800T	110% 7000T	108% 7200T	110% 6900T	85% 5800T	100% 6800T		
4	2	105% 8700T	110% 8500T	85% 6400T	90% 6900T	105% 8500T	110% 9000T	105% 8500T	102% 8200T	80% 6200T	90% 7500T	105% 8500T	110% 8500T		
5	2	110% 8500T	88% 7500T	70%* 6500T	80% 6500T	80% 6200T	85% 6000T	105% 7500T	105% 8200T	115% 8000T	105% 7900T				
6	2	110% 8600T	88% 8500T	70% 7000T	75% 5500T	75% 6000T	70% 6000T	95% 8200T	110% 8000T	110% 8100T	105% 7900T				
7	2	110% 8600T	88% 8500T	78% 4900T	69% 5000T	65%* 5000T	68% 6000T	62% 5500T	100% 6200T	110% 8500T	105% 8200T	100% 7800T			

8 (control)	2	110% 8250T	105% 7800T	105% 8200T	100% 7900T	105% 8100T	110% 8600T	105% 8000T	100% 7900T	102% 8150T	105% 7800T	105% 8100T	105% 8250T
9	2	95% 8700T	105% 10000T	110% 9000T	105% 8000T	110% 80000	108% 8500T	105% 8300T	105% 8200T	100% 7500T	102% 7800T	110% 8300T	105% 8000T
10	2	95% 8500T	100% 9000T	95% 9000T	100% 8700T	105% 8200T	100% 8000T	110% 8200T	100% 7800T	110% 7500T	105% 8200T	105% 9000T	105% 9200T

The letter 'T' is used in place of the last three zeros of the red cell count.

The hemoglobin is expressed in percentages as calculated from readings with the Dare hemoglobinometer.

• *Bartonella muris* bodies occasionally seen on red cells.

*muris* anemia with marked fatty changes in the liver, congested spleen and anemia of the organs. The blood had a watery consistency.

This is the first instance to our knowledge of the production of *Bartonella muris* anemia in the guinea pig. The strain recovered from the guinea pig was infectious for young rats.

The organism is non-pathogenic for adult guinea pigs.

#### *Infection of Young White Mice with Bartonella muris Cultures*

The susceptibility of white mice for *Bartonella muris* infection has been reported by Adler (7) and by Lwoff and Vaucel (3).

Four white mice were injected intraperitoneally with 0.3 cc. of a *Bartonella muris* culture. In three instances occasional *Bartonella* bodies were seen on the red cells after a period of 3 to 6 days. They occurred primarily in the red cells. In one instance a severe anemia occurred on the 5th day following the injection of the culture. The count dropped to 2,700,000 red cells per c.mm. and the hemoglobin to 29 per cent. The culture of the blood was positive for *Bartonella muris* on the 6th day.

#### *Infection of Adult Wistar Splenectomized Rats of Non-Carrier Stock with Bartonella muris Culture*

The Wistar stock are non-carriers of *Bartonella muris* infection. The adult Wistar rat is markedly resistant to infection, though it may be infected by the injection of large amounts of blood of anemic splenectomized rats (8). The splenectomized adult Wistar rat is very susceptible to infection with *Bartonella muris* anemia (9). The rats used in the test recorded were of Wistar stock, transported from the Wistar Institute to the laboratory of the Montefiore Hospital Country Sanatorium, 50 miles from the city, and bred there, out of all possible contact with infected stock. They were sent to our laboratory at the time of use and isolated.

With the early cultures a definite anemia was not produced in splenectomized adult Wistar rats though the organism was recovered from the blood stream several days following the injection of the culture. After 10 subcultures and 1 animal passage, however, the organism produced a mild anemia in five of seven adult rats of the Wistar stock after an incubation period of 3 to 5 days.

The hemoglobin dropped to 60 per cent and the red cells to 4,200,000 from 8,500,000 per c.mm. (see Table V). The organism was recovered from the blood. The white cell count rose from 10,500 to 65,000. This marked leukocytosis is characteristic of spontaneous *Bartonella muris* anemia. Occasional *Bartonella muris* bodies were found in red cells. They were not found in all cases and were never numerous. When large amounts of a culture were injected into Wistar unsplenectomized adult rats, they died within 24 hours of a severe toxemia. The organs showed some congestion. Adult unsplenectomized rats of carrier stock remain unaffected by injections of the organism.

### Serological Tests

Agglutination tests with homologous sera of rabbits repeatedly injected with 1 cc. of a heavy suspension of washings from blood agar slants proved negative.

Two rabbits were injected intravenously at 5 day intervals with 1 cc. of a heavy suspension of washings of cultures on blood agar. 5 days after the third injection the serum was tested against a suspension of living *Bartonella muris*. The suspension was obtained by repeatedly washing the bacteria obtained from blood agar slants. After four washings with distilled water the bacteria were resuspended in distilled water and utilized in agglutination tests. Negative results were obtained even in high concentration of the serum. Negative results were obtained with serum of infected rats and with serum of rats spontaneously infected with *Bartonella muris*.

Complement fixation tests were carried out using the bacterial suspension as antigen.

One-quarter of the anticomplementary amount of the antigen was used in the test. The serum of carrier rats, of spontaneously infected splenectomized rats, of rats infected with *Bartonella muris* cultures, of Wistar non-carrier rats and of homologous rabbit serum were tested. Positive fixation of complement was obtained in homologous rabbit serum and in anemic rats in very low dilutions (1/40). Similar results were obtained with antigen prepared by prolonged aqueous extraction of bacterial suspensions in a Soxhlet apparatus.

### DISCUSSION

*Bartonella muris* and *Bartonella bacilliformis* resemble each other much both morphologically and culturally. Noguchi isolated *Bartonella bacilliformis* from the blood of a patient suffering with Oroya fever and reproduced the disease in monkeys (1). He established the identity of verruga peruana and Oroya fever by producing Oroya

infection in a monkey with macerated material of a verruga nodule and reisolated *Bartonella bacilliformis* from the blood of the infected monkey (10, 11). In our experience *Bartonella muris* is somewhat less delicate in appearance than *Bartonella bacilliformis*<sup>3</sup> and stains more deeply. The growth on leptospira medium is definitely more luxuriant and the growth in subcultures appears in 48 hours, whereas *Bartonella bacilliformis* appears in 8 to 10 days. The growth on blood hormone agar during the first few days is similar to that of *Bartonella bacilliformis* but the *muris* colonies soon coalesce and form a tenacious film.

The similarity of *Bartonella muris* anemia in the rat and Oroya fever of human beings and the morphological and cultural characteristics of the two organisms suggest a close relationship between *Bartonella muris* and *Bartonella bacilliformis*. Further work will be undertaken to determine the possible rôle of the rat in the epidemiology of Oroya fever and verruga peruana.

#### SUMMARY

1. *Bartonella muris* has been isolated in pure culture on Noguchi's leptospira medium from the blood of the splenectomized adult rat suffering with *Bartonella muris* anemia.

2. *Bartonella muris* is a small, actively motile, Gram-negative bacillus. It grows best on media containing blood and on Noguchi's leptospira medium. The optimal temperature for growth is 20–25°C. It produces neither gas nor acid on media containing sugars. It does not hemolyze blood in artificial media. Viability of the cultures in leptospira media was maintained for 36 days.

3. With this culture a severe anemia was produced in rats weighing 30 gm., with the occasional appearance of *Bartonella muris* bodies on the red cells. The anemia occurred within 24 hours, and lasted for 3 to 5 days with recovery of the rat. *Bartonella muris* was recovered in pure culture from the blood of these animals. The blood of these rats was infectious for other 30 gm. rats.

4. 3 week old rabbits were infected with cultures of *Bartonella muris*, a severe anemia resulting after an incubation period of 48 hours. The

<sup>3</sup> We are indebted to Dr. Peter Olitsky of The Rockefeller Institute for Medical Research for a culture of *Bartonella bacilliformis*.

organism was recovered from the blood on the 5th day after injection. *Bartonella muris* is non-pathogenic for adult rabbits.

5. A severe anemia was produced in young guinea pigs with cultures of *Bartonella muris* within 48 hours. The organism was recovered on the 2nd and 5th days after injection. Postmortem examination revealed changes in the organs similar to those found in splenectomized rats suffering with spontaneous *Bartonella muris* anemia.

6. The infection was reproduced in white mice. In one instance a severe anemia developed on the 5th and 6th days. The organism was recovered on the 6th day.

7. The anemia was produced in splenectomized adult rats of non-carrier stock. The organism was recovered from the blood stream of these rats. A marked leukocytosis was noted (65,000) at the peak of the anemia as is found in the spontaneous disease in infected splenectomized adult rats.

8. Serological tests have thus far failed to demonstrate the production of agglutinins, though complement-fixing antibodies are present in homologous sera in low dilutions.

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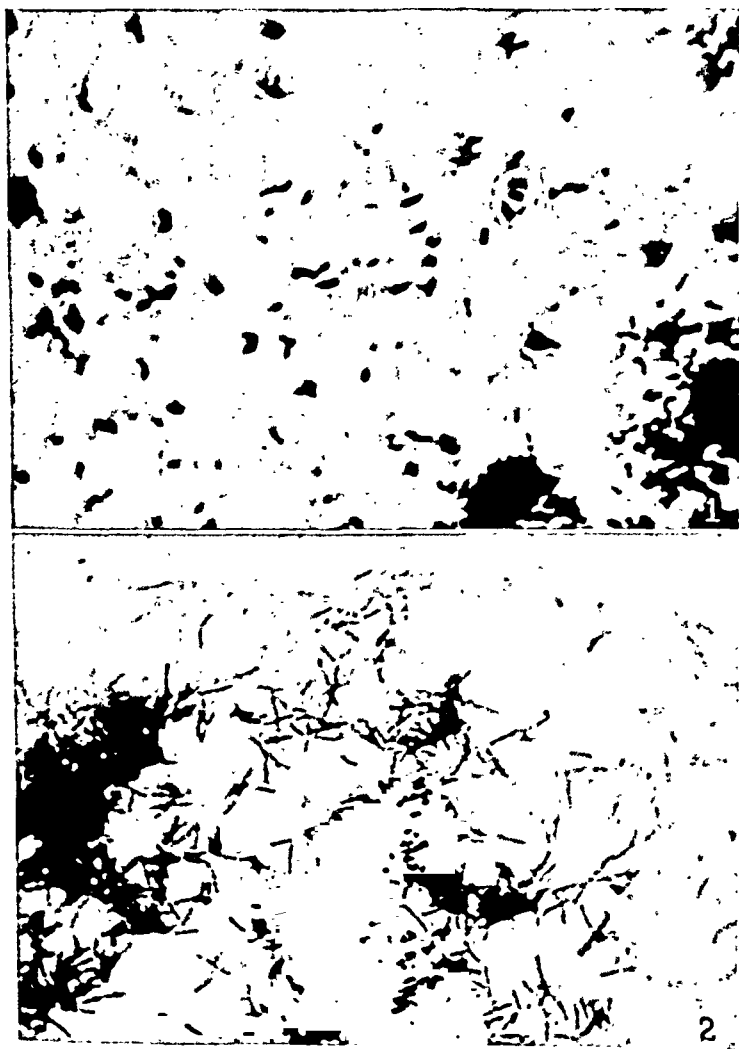
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#### EXPLANATION OF PLATE 36

FIG. 1. *Bartonella muris* growth in leptospira medium. Gram stain.  $\times 1200$ .

FIG. 2. *Bartonella muris* growth on blood hormone agar. Gram stain.  $\times 1200$ .





(Marmorston-Gottesman and Perl: Etiology of *Bartter's* renal anemia)





STUDIES ON *BARTONELLA MURIS* ANEMIA

VI. A LIPOID EXTRACT OF THE SPLEEN THAT PREVENTS *BARTONELLA MURIS* ANEMIA IN SPLENECTOMIZED ALBINO RATS

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In previous work (1), the authors have demonstrated that minute splenic autoplasmic transplants made 7 weeks prior to splenectomy protect a large percentage of splenectomized rats against *Bartonella muris* anemia. A comparative histological study of the transplants of protected and unprotected rats revealed a regeneration of the pulp cells in the protected rats, and an exhaustion destruction of the pulp in the unprotected rats. This supported the hypothesis that the reticular and endothelial cells of the pulp of the spleen elaborate some internal secretory substance having an influence to prevent *Bartonella* infection. Lauda and Flaum (2) found that rats joined by parabiosis are protected against *Bartonella muris* anemia if the spleen of only one animal is removed. From their experiments these investigators concluded that the protective action is due to a hormonal substance produced in the spleen.

Unsuccessful attempts have been made by numerous investigators to demonstrate some substance in the spleen which would replace this organ in protecting adult splenectomized rats against *Bartonella muris* anemia. During the past 3 years we have made many attempts to obtain such an extract. Feeding raw or cooked spleen of ox, calf, pig, or rat had no effect on the course of the disease. Recently, however, lipid extracts of the spleen have been prepared which possess the property of protecting splenectomized adult albino rats against *Bartonella muris* anemia in a large percentage of instances.

Since the anemia is more severe in the male, only male rats were used in testing the extracts. In no instance among 440 male rats of carrier stock used for studies of *Bartonella muris* anemia during the

past 3 years, has the anemia failed to develop following splenectomy. Similar observations on the prevalence of the disease in the male have been made by Ederle and Kriech (3). It is plain that the spleen plays a specific rôle in the protective mechanism of the body to the infection. Rats of carrier stock are infected early in life, they recover, and remain carriers of the infection.<sup>1</sup> Subsequent splenectomy is followed by a recurrence of the infection with the development of a severe anemia.

### *Preparation of the Extract*

The method of extraction is the same as that used by Hartman (10) in the preparation of the cortical suprarenal hormone. Ox spleens were obtained from freshly killed animals, packed in ice, and immediately transported to the laboratory. 10 pounds of spleen were extracted at a time.

The fat and as much of the capsule as possible is stripped off, and the pulp is ground in a meat grinder. The trimming and grinding is done by artificial light and the material kept cold. The ground material is placed in large round bottom flasks and 1.5 liters of peroxide-free ether is added for each kilo of spleen. The ether extraction is done in the dark in an atmosphere of CO<sub>2</sub>, and the extraction is assisted by a rotary motion of 15 to 20 rotations per minute. More rapid agitation may result in emulsification. If possible the temperature of the room should be kept below 10–12°C. The flasks are shaken for a period of 3 hours, and allowed to stand overnight. The ether is decanted, and rapidly filtered through coarse filter paper. The ether extraction is repeated for a total of 3 extractions. The ether removed is evaporated at a temperature of 15°C. *in vacuo*. All the ether fractions are added together. It is best to evaporate the ether immediately after decanting and to add the new fractions to the residue. The residue of the ether extraction is now extracted with 95 per cent alcohol. 100 cc. of 95 per cent alcohol is used for each kilo of spleen extracted. The alcohol is warmed to 60°C. for a period of 30 minutes, the flask being shaken every 10 or 15 minutes. The flask is slowly cooled to ice temperature and the alcohol extract decanted. The same quantity of alcohol is again added, and extraction repeated 4 times, to insure thorough extraction.

The alcohol fractions are pooled, and chilled to –10°C. for 2 hours in chopped ice to which CaCl<sub>2</sub> has been added. This process causes the precipitation of large amounts of cerebrosides. The chilled material is then rapidly filtered in the ice box, and the filtrate is now a clear brownish yellow liquid. It is essential that filtration be done rapidly and in the cold. If the filtrate is not clear the chilling process should be repeated before proceeding. The spleen contains large amounts of inert lipoids and their removal is attended with difficulty. The alcohol filtrate

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<sup>1</sup> See previous publications of the authors on this subject (4–8). For a review of the literature to 1930 see Lauda (9).

is now evaporated to one-third its volume *in vacuo* at a temperature of 45–50°C. The alcohol fractions will keep for several weeks. The reduced volume of alcohol is again chilled, and filtered in the manner outlined above. The filtrate is now evaporated to dryness *in vacuo* at 45–50°C. The residue is repeatedly extracted with a small amount of ether. More complete extraction is obtained by agitation and scraping with a rubber "policeman" during the process. This procedure is repeated 4 times with at least 10 cc. of ether and is carried out in the dark room by red light, the flask being kept on ice during the procedure. The ether extractions are filtered through coarse and then fine filter paper, and evaporated to dryness *in vacuo* at a low temperature. Great difficulty will be encountered in this last step if there is a large amount of alcohol residue. In that case, in order to eliminate inert material, it is advisable to do another alcohol extraction with subsequent chilling and filtering.

The final residue of the last ether extraction is a small amount of brownish oily material. If the extractions have been carefully carried out not more than a few drops of this material remain. It is thoroughly emulsified with distilled water (50 cc. for 5000 gm.) in the required amount, and filtered through a Seitz filter. The resultant aqueous extract has a yellowish tinge. It is brought up to isotonicity, and preserved with 0.1 per cent of benzoic acid. Physiological salt solution may be used for the emulsification in place of distilled water. 1 cc. of the extract is equivalent to 100 gm. of spleen. The aqueous extract will keep for several weeks if a preservative is added.

Numerous attempts to obtain larger yields were made by suspending the oily residue of the final ether extraction in olive oil. The resultant emulsion produced severe peritoneal irritation when injected intraperitoneally into rats. An attempt to fractionate the final ether-soluble material into acetone-soluble and insoluble fractions was made in the following manner: An excess of acetone was added to the final ether extract until no further precipitation occurred. The precipitate was filtered off and dissolved in benzene. The filtrate was evaporated and dried *in vacuo*, taken up in water, and passed through a Seitz filter. The benzene extract of the acetone-insoluble fraction was evaporated to dryness *in vacuo* and taken up in water. When tested neither the acetone-soluble nor the acetone-insoluble fraction showed evidence of potency.

Extracts of fresh spleen, made by extraction either with acetone or with HCl, proved unsuccessful. On analysis, the final aqueous lipid extract gave no reactions for either protein or carbohydrate. Traces of nitrogen were detectable (Kjeldahl). No copper or iron could be detected by qualitative tests.

#### EXPERIMENTAL DATA

Twenty-nine splenectomized male albino rats of carrier stock were tested with the lipid extract of spleen. Of these, 8 were 6 to 8 weeks of age and 21 were 3 to 5 months old. The extract was administered twice daily intraperitoneally in

amounts of 0.5 cc. The injections were started 24 hours prior to splenectomy. Hemoglobin estimations with the Dare hemoglobinometer, red blood cell counts, and smears were made daily. A rat was considered completely protected if the blood count and hemoglobin did not show greater variation than is observed in normal rats, and *Bartonella* bodies were not found on the red cells. The animals were under observation for at least 1 month following splenectomy, to eliminate the possibility of a delayed appearance of the anemia.<sup>2</sup> All the rats were of *Bartonella muris* carrier stock, raised in the laboratory, and used for studies of this anemia during a period of several years.

TABLE I

*The Protective Action of an Aqueous Lipoid Extract\* of Spleen against Bartonella muris Anemia in Male Splenectomized Rats of Carrier Stock*

No. of rats	Age	Completely protected†	Unprotected	Protected per cent
Treated				
8	6-8 wks.	3	5	37
21	3-5 mos.	14	7	66
Controls				
0.5 cc. saline twice daily				
40	3-5 mos.	0	40	0
Theelin (ovarian follicular hormone) 10 units per day				
12	3-5 mos.	0	12	0

\* The extract was administered twice daily intraperitoneally in amounts of 0.5 cc. (1 cc. of the extract is equivalent to 100 gm. of spleen).

† A rat was considered completely protected if the blood count and hemoglobin did not show greater daily variations than is found in normal rats, and if *Bartonella* bodies were not found in the red cells.

In the first group of immature rats, complete protection against *Bartonella muris* infection and anemia was obtained in 3 out of 8 rats. In the second group of mature rats, 14 out of 21 rats were completely protected. Of 40 male splenectomized rats injected daily with physiological salt solution, all developed *Bartonella muris* anemia. As a

<sup>2</sup> In the control splenectomized untreated rats the anemia develops in most instances during the 1st week.

further control, the ovarian follicular hormone, Theelin (Parke, Davis and Co.) an aqueous extract of the crystalline hormone, was administered to 12 adult male splenectomized rats in amounts of 10 rat units per day injected intraperitoneally. No protection against *Bartonella muris* anemia was observed.

Rats of carrier stock between the ages of 6 and 8 weeks, with the spleen intact, suffer from a severe infection of *Bartonella muris* with little or no anemia. This is manifested by the occasional appearance of *Bartonella muris* bodies on the blood cells and the marked hyperplasia and congestion of the spleen. Protection in such animals against anemia following splenectomy by an extract of the spleen is, therefore, less effective. In the adult, the infection is latent and the spleen shows little evidence of hyperplasia and congestion. In these rats protection was obtained in a large percentage of instances.

#### DISCUSSION

Numerous investigators have found that liver extracts potent in the treatment of pernicious anemia have no effect on the course of *Bartonella muris* anemia. Recently Ederle and Kriech (3) have studied the effects of injections of a commercial extract of liver, administered subcutaneously in amounts equivalent to 2 gm. of liver a day. They observed that the anemia in the treated rats was less severe, and the mortality was less than in the control group. On the other hand, Vedder (11) obtained entirely negative results with liver therapy.

The literature contains numerous references to the use of spleen extracts in various diseases, but interpretation of such results is difficult. There are many evidences of splenic function, but their significance is not plain. The body of most animals contains accessory splenoid tissue in varying amounts. The rat possesses less hemolymph tissue than other mammals (12) and a much larger spleen, and the removal of the spleen in this animal seems to have an unusually marked effect on resistance. The specific relation between *Bartonella muris* anemia in the rat and the function of the spleen offers a possible index of function of the pulp tissue of this organ.

The protective action of a splenic extract against *Bartonella muris* anemia in the splenectomized adult male rat upholds the hypothesis that such an extract contains a specific internal secretory substance.

## SUMMARY

An aqueous lipid extract of ox spleen was prepared which protects adult male albino rats of carrier stock in a large percentage of instances against *Bartonella muris* anemia following splenectomy. It is suggested that the extract contains a specific hormonal substance.

We wish to thank Dr. David Marine for his suggestions and criticism throughout the course of this work.

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## STUDIES ON BARTONELLA MURIS ANEMIA

## VII. THE PROTECTIVE ACTION OF COPPER AND IRON AGAINST BARTONELLA MURIS ANEMIA

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The extensive experiments of Hart, Steenboch, and Elvehjem and their coworkers (1-5), Beard and Myers and their associates (6-11), and others have established the importance of copper in hemoglobin formation. Young rats fed on a diet of milk deficient in copper develop an anemia within 6 to 8 weeks which can readily be prevented by the administration of small quantities of copper in the diet. A minimal requisite of 0.025 mg. of elemental copper per rat per day was determined by these investigators. Since albino rats are subject to *Bartonella muris* infection spontaneously during the early weeks following weaning, it was thought that the milk anemia of the rat may be complicated by infection with *Bartonella muris*. The prophylactic effect of copper in the milk anemia of rats suggested its trial in *Bartonella muris* anemia of splenectomized adult rats.

The rats used in these experiments were all carriers of *Bartonella muris*. They were of stock raised in our laboratory for many years and maintained under constant environmental and dietary conditions. The diet for the past 10 years has consisted of 15 gm. per rat per day of a mixture composed of hominy 100 parts, rolled oats 25 parts, fine meat and bone 25 parts, dried skim-milk 16 parts, and salt  $1\frac{1}{2}$  parts. Twice a week the rats received whole milk and bread *ad lib.* and greens (lettuce leaves). The exact copper content of this diet was difficult to estimate but the food mixture was found to contain about 0.025 mg. of elemental copper per 15 gm. of food. In the experiments reported the copper was added in the form of copper sulfate in doses equivalent to 0.1 mg. of elemental copper per day. Lactose was used as a vehicle. The iron was added in the form of iron ammonium citrate in doses equivalent to 1 mg. of elemental iron per day.

Following splenectomy, daily hemoglobin estimations and smears were made on all the rats and the red cell counts were made every other day. In determining



the protective action of copper and iron supplements to the normal diet, against *Bartonella muris* anemia in splenectomized rats, it was necessary that the animals should be observed for a period of 1 month to exclude a delayed appearance of anemia following splenectomy. If the hemoglobin percentage and the red cell count did not fluctuate more than is usual in the normal rats and *Bartonella* bodies were not present or only sparsely found, it was considered that complete protection against the infection and the anemia was obtained.

In the experiments reported both immature and mature rats were used. The experiments were divided into three groups. In the first group of experiments, the copper and iron supplements to the diet were given for a period of 2 days prior to and 1 month subsequent to splenectomy. In the second group, the copper and iron supplements to the diet were given during a period of 9 to 12 days prior to and 1 month subsequent to splenectomy. In the third group, copper and iron supplements to the diet were given for a period of 2 months prior to and 1 month subsequent to splenectomy.

#### *The Effect on Bartonella muris Anemia of Copper and Iron Supplements to an Adequate Diet*

In the first group of experiments, 18 rats, 3 to 4 months old, were used. Four received daily supplements of iron in the form of iron ammonium citrate in amounts equivalent to 1 mg. of elemental iron per day, 8 received additions of copper as copper sulfate in amounts equivalent to 0.1 mg. of elemental copper per day, and 6 received both copper and iron additions daily. The supplements were commenced 2 days prior to splenectomy and continued thereafter.

All the animals developed severe *Bartonella muris* anemia. No protective action of the copper and iron was observed in this group. (See Table I.)

In the second group of experiments, 46 rats were fed diets supplemented with copper, iron, or copper and iron during a period of 9 to 12 days prior to and 1 month subsequent to splenectomy. Thirty untreated splenectomized adult rats were used as controls during the same period.

Of the treated rats, 20 received supplements of copper alone in amounts equivalent to 0.1 mg. of copper per day.<sup>1</sup> Of these, 8 were immature rats, and 12 were 4 months of age. Of the group of immature rats 3, or 37 per cent, were completely protected against *Bartonella muris* infection and failed to develop any evidence of anemia following splenectomy. Of the mature animals 9, or 75 per cent, were protected against the anemia. Fourteen rats received supplements of iron in the form of iron ammonium citrate in amounts of 1 mg. of iron per rat per day. Of

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<sup>1</sup> It was found that intraperitoneal injections of copper as copper acetate in physiological salt solution are as effective as feeding the copper.

these, 4 were immature rats and 10 mature rats. Of the young rats only 1 was protected. Of the mature rats 5 were protected. (See Table I.)

Twelve rats received both iron and copper supplements in the same amounts as used in the previous experiments (0.1 mg. copper and 1 mg. iron per rat per day) during 9 to 12 days prior to and 1 month subsequent to splenectomy. Of 4 young

TABLE I

*Effect on the Incidence of Bartonella muris Anemia Following Splenectomy of Additions of Copper and Iron to an Adequate Diet*  
Summary of Experiments

No. of rats	Age	Addition to diet	Feeding commenced prior to splenectomy	Protected	Unprotected	Protected
			days			per cent
4	3-4 mos.	Fe	2	0	4	0
8	3-4 mos.	Cu	2	0	8	0
6	3-4 mos.	Cu and Fe	2	0	6	0
8	9 wks.	Cu	9-12	3	5	37
12	4 mos.	Cu	9-12	9	3	75
4	9 wks.	Fe	9-12	1	3	25
10	4 mos.	Fe	9-12	5	5	50
4	9 wks.	Cu and Fe	9-12	4	0	100
8	4 mos.	Cu and Fe	9-12	6	2	75
10	9 wks.	Control	—	0	10	0
20	3-5 mos.	Control	—	0	20	0
11	4 mos. (at time of operation)	Cu	60-80	7	4	63
8	4 mos. (at time of operation)	Fe	60-80	2	6	25
8	4 mos. (at time of operation)	Cu and Fe	60-80	3	5	36

rats treated, all were protected. Of the 8 mature rats, 6, or 75 per cent, were completely protected against the *Bartonella* infection and did not develop any anemia.

Of the 30 control splenectomized adult rats observed in the same period, all developed a severe anemia.

From these experiments (see Table I) it is evident that copper and iron protect a large percentage of rats against the *Bartonella* infection following splenectomy if the copper and iron supplements are given during a period of approximately 10 days prior to splenectomy and continued thereafter. Copper is definitely more effective than

iron, and copper and iron slightly more effective than copper alone. In the groups fed diets supplemented by copper alone or iron alone the adult rats were protected in twice as many instances as were the immature rats, doubtless because of the fact that the severity of the infection with *Bartonella muris* (without anemia) is much greater in the immature rat with intact spleen than in the adult rat in which the infection is entirely latent. This is manifested by the reaction of the spleen. The percentage weight of the spleen to the body weight of immature rats of carrier stock is much greater than that of mature rats. The spleen in the young rat of *Bartonella muris* carrier stock shows the histologic changes of congestion of the pulp and hyperplasia of the follicles.

Copper protects 75 per cent of adult rats against *Bartonella* anemia following splenectomy if it is added as a supplement to an adequate diet for a period of 10 days prior to splenectomy.

In the third group of experiments, 27 rats received supplements of copper and iron to the normal diet during a period of 2 months prior to and 1 month subsequent to splenectomy. Six untreated rats fed on the normal diet were used as controls. All the rats were 7 to 8 weeks of age at the time the supplemented feedings were commenced and about 4 months of age when splenectomized. All the controls developed a severe infection following splenectomy. Of the treated rats, 11 received supplements of copper alone in amounts equivalent to 0.1 mg. of elemental copper per rat per day. Of these, 7, or 63 per cent, were completely protected against the infection and the anemia following splenectomy. Eight rats received supplements of iron in amounts equivalent to 1 mg. of elemental iron per rat per day. Of these, 2 were protected. Eight received both copper and iron supplements in amounts equivalent to 0.1 mg. and 1 mg. respectively of the elemental metal per rat per day. Of these, 3 were protected.

From these experiments (see Table I), it is evident that supplements of copper to the diet given during a long period of time protected a considerable number of rats against *Bartonella muris* anemia following splenectomy. The addition of iron resulted in protection in only a few instances and the addition of both copper and iron resulted in less protection than the addition of copper alone. The protective action of supplements of copper and iron to the diet, when these are added during a period of 2 months preceding splenectomy, is not as great as that of copper and iron when added for a period of 10 days prior to splenectomy.

It is of considerable interest that in two instances the rats fed copper alone for 2 months developed a slight anemia several days prior to splenectomy. At operation the percentage weight of the spleen to body weight of the rat (0.9 per cent) was considerably greater than the average for this age period (0.27 per cent). Following removal of the spleen the anemia cleared up and the rat remained free of infection during the period of observation of 1 month. The large amount of copper may have resulted in injury to the pulp cells of the spleen. This was manifested by a recurrence of the *Bartonella muris* infection. That the removal of the spleen in these cases resulted in a lessening of the infection and disappearance of the anemia is a unique experience in our observations of *Bartonella muris* anemia. The fact that rats fed diets supplemented with iron, or copper and iron for a period of 2 months prior to splenectomy were not protected was probably the result of cellular injury resulting from excess storage of the metals—primarily the iron. The liver and spleen were found heavily laden with iron pigment. The total quantity of copper salt received by the treated rats to the day of splenectomy was equivalent to 6 to 8 mg. of elemental copper per rat and of iron salt, 60 to 80 mg. of elemental iron per rat.

Cannon and McClelland (13), Haendel and Haagen (14), Friedberg (15), Rosenthal and Zohmann (16), and Judenik (17) observed that repeated injections of suspensions of India ink or other inert colloidal substances may be followed either by spontaneous *Bartonella muris* anemia in the rat with intact spleen or by an increased susceptibility to the injection of blood of an anemic splenectomized rat (superinfection). They attribute this depression in the acquired resistance to *Bartonella muris* infection to the cellular injury resulting from blocking of the reticulo-endothelial system. The effect obtained is similar to that which follows splenectomy.

#### DISCUSSION

The effect of the addition of small amounts of copper to an adequate normal diet during a period of a little more than a week to prevent *Bartonella muris* anemia in albino rats of carrier stock is of importance in relation to the physiologic utilization of copper in the body. The need for small amounts of copper in the production of hemoglobin has been demonstrated by work already referred to. Copper is essential as a catalytic oxidative agent in the formation of hemoglobin. From the present work it would seem to be an important substance in the maintenance of resistance to *Bartonella muris* anemia. The *Bartonella* infection occurs spontaneously in rats of carrier stock between the 5th and 7th weeks.<sup>2</sup> It remains latent but the acquired resistance

<sup>2</sup> McCarrison and Singh (18) recently observed *Bartonella muris* bodies on the red cells in new-born rats of normal mothers during the first 4 days of life in about 20

established on the first invasion of the animal can be broken down by splenectomy. The anemia that follows is severe and striking, and results in a mortality of at least 30 per cent. Protection against this infectious anemia may be due either to a direct toxic action of copper and iron on the *Bartonella muris*, or to an indirect action intimately related to splenic function. In several instances we have observed, some weeks after copper had been discontinued, a recurrence of *Bartonella* infection with anemia in rats splenectomized and protected for 1 month by copper. It seems probable that the copper exerts an indirect protective effect rather than a direct toxic effect on the bacterium.

Feldt and Schott (19) have maintained that the chemotherapeutic action of salvarsan against spirochetal infections is due to an indirect action on the reticulo-endothelial cells. They found that when these cells were blocked with metallic colloids and splenectomy had been done in mice infected with *Spirocheta febris recurrentis*, the animals could not be cured by chemotherapeutic agents otherwise specific in their action. This is in accord with our observations in experiments in which an excess of copper and iron was fed to rats over a period of 60 to 80 days. The excess storage of the metallic substances may be thought to have injured the reticulo-endothelial cells and thus to have prevented the action of the copper and iron against the *Bartonella muris* anemia. The difference between the toxic dose of copper and the physiological requirement of this metal is, however, considerable.

In a previous study we have been able to procure from the spleen a substance (20) which when injected into adult albino rats, beginning on the day of splenectomy, protects in a large percentage of instances against *Bartonella muris* anemia. The extract was prepared in the manner of Hartman's suprarenal cortical extract. The specific relation of the *Bartonella muris* anemia in the adult rat to splenic function is well established. That an extract can replace the spleen in protecting the animal against this anemia strongly suggests that the specific protective function of the spleen is due to a substance secreted by the pulp cells which in some manner influences cellular response to infection. This extract contains no protein material, and no trace of either copper or iron. We have suggested that it contains a specific hormonal substance elaborated by the spleen.

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per cent of instances. Apparently in some cases infection with *Bartonella muris* may occur at birth.

It is possible that copper exerts its effect by influencing the oxidative processes involved in cellular activity<sup>3</sup> and in this way affects the resistance of the cell to toxic substances.

The literature on the relation of the spleen to iron metabolism is exhaustively and critically reviewed by Lauda (22). He states that the experimental evidence reported, is inconclusive in demonstrating any relationship between the physiology of the spleen, and either the storage or utilization of iron. The relation of copper to the spleen may be somewhat analogous to the relation of calcium to the parathyroid gland. The small amount of copper in the normal diet of the rat is insufficient for the needs of the rat in the absence of the splenic hormone. An excess of the metallic element in the diet may compensate for a deficiency of the hormone. Further, the copper, administered as an inorganic salt cannot be utilized at once but must be converted into a form that is utilizable by the body in the mechanism of resistance to *Bartonella muris* anemia.

Cunningham (23), in a careful study of the relative amounts of copper in various animal and plant tissues, found that the rat has less copper in its organs than any other animal studied. The copper ingested is stored primarily in the liver (21). Corper (24) found very little copper stored in any other organ than the liver, and only traces were recovered from the spleen. The liver of the rat contains 1/20th to 1/30th the percentage weight of copper found in the liver of the rabbit, the guinea pig, the sheep, or the ox (21). This is probably a result of great differences in the copper content of the food of these animals. The fodder of the guinea pig and the rabbit contains large amounts of green vegetables very rich in copper (22). It may be that the resistance of the adult guinea pig and the rabbit to *Bartonella muris* infection, which exists whether the spleen is present or not (25), is dependent on the high copper content of the diet. This would explain the susceptibility of the suckling young of these species, in which copper is as yet not stored in significant amounts (25).

These studies suggest a possible importance of dietary copper and of iron in relation to resistance to infectious anemias in human beings.

<sup>3</sup> The influence of copper on oxidative processes has been demonstrated by Voegtlin (21) in the case of glutathione. Glutathione combined with copper oxidized 1000 per cent more rapidly than glutathione not prepared in this way.

We plan to investigate the relation of dietary copper to various types of infection.

There is considerable literature on the administration of copper salts in the treatment of infectious diseases, but most of it is difficult to evaluate, and the reports are contradictory or the experiments poorly controlled. Good results have been reported from its use in oidiomycosis, actinomycosis, and sporotrichosis by Bevan (26). Its use in tuberculosis therapeutically, both experimentally and in patients, receives some support from the work of von Linden (27), Meissen (28), and Straus (29) but as Corper points out, their work is inadequately controlled. Corper could find no evidence of a therapeutic action of copper in experimental tuberculosis in guinea pigs, but he was not essentially concerned with the *physiological* rôle of copper in the mechanism of resistance. The animals employed in his experiments (guinea pigs and rabbits) have large stores of copper as result of a diet naturally rich in copper. An excess of copper added to their diet might well have a detrimental effect on cellular physiology. A relative paucity of copper in the diet, as normally occurs in the care of the rat, may, in the absence of a splenic hormone influencing infection, result in a diminution of resistance to certain types thereof. Additions of copper to the diet of these rats may raise their resistance. It does not follow, however, that an excess of copper added to a diet containing foods naturally rich in copper would necessarily increase the resistance of the host.

#### SUMMARY

The effect on *Bartonella muris* anemia of adding copper or iron or both to an adequate diet was studied.

The addition to the diet of copper (0.1 mg. per rat per day), or iron (1 mg. per day), or both during a period of 2 days prior to splenectomy and 1 month subsequent thereto failed to protect adult albino rats against *Bartonella muris* anemia.

The addition of copper to an adequate diet for a period of 10 days prior to splenectomy and 1 month subsequent thereto protected 75 per cent of the rats against the anemia.

The addition of iron (1 mg. per rat per day) for a period of 10 days prior to and 1 month subsequent to splenectomy protected 50 per cent of the rats against this anemia.

The addition of both copper and iron for a period of 10 days prior to and 1 month subsequent to splenectomy protected 75 per cent of these rats against *Bartonella muris* anemia.

The addition of copper alone for a period of 2 months prior to and 1 month subsequent to splenectomy protected 63 per cent of the rats against *Bartonella muris* anemia.

The addition of iron, or of both copper and iron during a period of 2 months prior to splenectomy and 1 month subsequent thereto protected about one-third of the rats against *Bartonella muris* anemia.

#### CONCLUSIONS

Copper plays a rôle in the mechanism of resistance to *Bartonella muris* anemia in the rat. The small amount of the element in the ordinary diet of the rat is insufficient to protect the animal after splenectomy. An excess of copper, however, may give protection in the absence of the spleen. Its utilization would seem to be intimately associated with splenic function.

We wish to express our appreciation to Dr. David Marine for his advice and criticisms during the course of the work.

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## A TRANSMISSIBLE TUMOR-LIKE CONDITION IN RABBITS

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PLATES 37 TO 40

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This paper deals with a tumor-like condition in a wild cottontail rabbit (genus *Sylvilagus*) and its experimental transmission to domestic rabbits (genus *Oryctolagus*). The term "tumor" is used in its broadest sense to indicate a local swelling consisting of a mass of new tissue.

*Description of the Original Material*

Three prominent subcutaneous tumors were observed in a wild cottontail rabbit shot in Nov., 1931. The animal was taken immediately to the laboratory and autopsied. Two of the tumors, on the inner and upper aspect of the left hind foot, were egg-shaped and approximately 1.5 x 2 cm. in diameter. The other, which was somewhat larger, was on the left fore leg just below the shoulder. There were no overlying surface abrasions and, although the tumors seemed to be firmly attached to the skin, they were freely movable over the underlying solid skeletal structures.

On section each of the tumors was found to be white, firm, moist, and free from evident necrotic areas. They cut as though fibrous, the cut surface bulged, and the general appearance was that of a fibroma. There was nothing of significance found in the remainder of the autopsy. The condition of the animal appeared good, it was not in the least emaciated, and there were no evidences of metastases in the regional lymph nodes or the organs.

Pieces of each of the tumors were removed aseptically to use in inoculating laboratory rabbits and the remainder was stored in 50 per cent glycerol or put in fixative for histological study.

Histologically the three tumors seemed to be identical.

The main mass was composed of connective tissue cells; many of these resembled fibroblasts and were spindle or polygonal in shape with abundant cytoplasm and large round or oval nuclei. Most of the cells, however, were of the small thin connective tissue type with scant cytoplasm and thin spindle-shaped nuclei (Fig. 1). Mitotic figures were scarce. The arrangement of the cells was rather compact and irregular; in general the long axes were perpendicular to the base of the tumor.

Intercellular fibrils were quite abundant and largely collagenous in character. The blood supply was good and consisted of small vessels whose walls were usually of only endothelium thickness. Some of these vessels were densely cuffed by lymphocytes and plasma cells. Inflammatory cells were not numerous in the tumor substance and consisted largely of eosinophiles and lymphocytes. The base of the tumor, however, was composed of a mass of lymphocytes so densely packed that it almost resembled lymphoid tissue.

The thickened epithelium overlying the tumor presented a histological picture strikingly similar to that seen in molluscum contagiosum in man. Great bulbous masses of epithelial cells projected down from the epidermis into the substance of the tumor (Fig. 1). Many of the cells of both the overlying epidermis and the bulbous downward projections were greatly enlarged, the nuclei were pale and vesicular and the cytoplasm was vacuolated and distended by a granular eosinophilic mass in which frequently lay a more definite, discrete, round, eosinophilic inclusion. Such vacuolated and inclusion-containing cells were numerous.

The histological picture of these wild rabbit tumors resembled that of a spindle cell fibroma; it was atypical, however, in that the bases were densely infiltrated with lymphocytes, there was a perivascular round cell cuffing of some of the nutrient blood vessels, and the overlying epidermis showed a peculiar type of hyperplasia and degeneration.

### *Experimental Transmission*

No difficulty was encountered in transmitting the condition to domestic rabbits by pushing small bits of the original wild rabbit tumors under the skin with a trocar. However, the tumor could only be maintained in serial passage through domestic rabbits when young, actively growing tumors were used as a source of material for transfer and when, on the basis of the experience of Brown and Pearce (1), the testicle was used as the site for inoculation. It was found not only that tumors developed more readily there than in the subcutaneous tissue, but also that such tumors provided a better source of infectious material for maintaining the condition in serial passage.

The tumor-like condition has been transmitted, thus far, only to laboratory and wild cottontail rabbits. Guinea pigs, white rats, white mice, and chickens have resisted infection by the methods used.

### *Methods*

The clinical picture exhibited by rabbits in which the tumor has been experimentally produced became constant only after the third

serial passage when a satisfactory technique, as described below, had been developed.

As a source of infectious material, tumor-bearing testicle was used alone or mixed with subcutaneous tumor. These tissues were minced finely with scissors, then ground with sterile sand, and finally combined with physiological saline to make an approximately 5 per cent suspension. After this suspension had been allowed to stand undisturbed for 5 to 10 minutes, the supernatant fluid was decanted and used in making inoculations. While the etiological agent persists in the tumors for relatively long periods of time, the most satisfactory results were obtained by using 6 to 9 day old tumors in preparing the suspensions.

This suspension was introduced subcutaneously in dosages of 1 to 2 cc., or intratesticularly in dosages of 0.2 to 1 cc. Usually only one testicle was inoculated, leaving the other for comparison.

### *Routes of Infection*

When a fully infectious suspension was employed, local tumor formation regularly resulted from inoculation into one or both testicles or subcutaneously in any portion of the body.

When introduced intramuscularly the tumor-inducing agent gave positive results in only about one-third of the cases. These variable results were probably dependent upon whether the injection lodged directly in a muscle bundle or in connective tissue fasciae between muscle bundles. In the former case no tumor was formed while in the latter a large fibrous tumor infiltrating the surrounding muscle resulted.

When an infectious suspension was injected intracutaneously or was rubbed on the scarified skin the results were not constant; usually on the 4th day the epidermis became thickened, elevated, and hyperemic, and minute vesicle formation sometimes occurred. Slight thickening of the underlying subcutaneous tissue was seen in some cases. The condition disappeared after 3 or 4 days, usually leaving a thin, loosely adherent scab.

Inoculation intravenously, even when rather coarse suspensions and large dosages were employed, was without effect, except for the regular formation of a small nodule in the subcutaneous tissue of the ear at the point where the needle was inserted. Likewise, inoculation intraperitoneally with large dosages of demonstrably infectious material resulted in no evidence of tumor formation in the peritoneum or

elsewhere. Intracerebral inoculation has been negative in the few animals used.

Evidence that the condition is not contagious is offered by the fact that normal rabbits placed in the same pens with tumor-bearing rabbits remained free of tumor and were subsequently, after prolonged contact, still susceptible to infection by inoculation. Furthermore, a doe bred to a buck carrying a large tumor in one testicle developed no tumor and gave birth to a normal litter.

Evidences of a generalized disturbance as indicated by loss of appetite or fever were absent. Rabbits bearing large tumors sometimes showed a slight loss in weight which was promptly regained as the tumors regressed. Most of the animals were killed while still carrying large tumors, but among the 30 whose tumors were allowed to regress there was no evidence of illness at any time and no deaths.

### *Clinical Picture and Pathology of Experimental Disease*

*Subcutaneous Inoculation.*—In a rabbit inoculated subcutaneously the tissue in the region of inoculation was definitely thickened by the 3rd to the 5th day. The following day the thickening was more marked and its borders were more sharply circumscribed so that it began taking on the gross appearance of a tumor. From this point on to the 10th or 12th day after inoculation, increase in size was progressive and rapid. Early in its growth the tumor felt soft and rubbery and small firmer nodules could be palpated in its substance. As it increased in size, it became more solid and developed into a very firm, well circumscribed, usually lobulated tumor with a tense, sometimes glistening, overlying epithelium (Fig. 2). Usually it was freely movable over the underlying skeletal structures and seemed well encapsulated. The eventual size of the tumor varied somewhat depending apparently on such factors as size of dose administered, amount of the inciting agent present in the infectious suspension, age of the tumor used in preparing the suspension, and factors relating to the inoculated rabbit itself. However, a tumor 4 x 6 cm. and 1.5 to 2 cm. in thickness was average. The tumor developed by deepening and filling up the original area of subcutaneous thickening with very little increase in the lateral diameters. When more than one subcutaneous site in a rabbit was inoculated simultaneously, each area developed a tumor which seemed to be uninfluenced by the presence of the others.

Examined when at its maximum size on from the 10th to the 12th day after inoculation, the cut surface of a subcutaneous tumor was white or pinkish white and bulged slightly (Fig. 3). It was moist but not edematous. Pieces of tumor were difficult to grasp with forceps because of their firm rubbery consistency. On section some tumors exhibited two distinct layers of approximately the same thickness held together by a thin layer of rather loose areolar tissue. The upper

layer was continuous with the overlying epidermis while the lower was movable both over the underlying muscle and in relation to the upper layer of the tumor. Sometimes pea-sized or larger nodules of the same firm consistency were a part of these layers of new tissue. Other subcutaneous tumors failed to exhibit a two-layer appearance on cut section and instead were composed of one solid mass of glistening white tissue or groups of closely packed nodules of the same general appearance.

After reaching a maximum size on the 10th to the 12th day, the course of a subcutaneous tumor was variable. Occasionally it persisted with no apparent change in size or consistency for periods ranging from 10 to 15 days, but usually, after 2 or 3 days, a rapid regression took place. If the tumor had reached a size great enough to cause an ischemia of the overlying epithelium with consequent gangrene, regression was more rapid and accomplished partly by sloughing of the gangrenous epithelium and its underlying tumor tissue. All subcutaneous tumors observed in laboratory rabbits have eventually completely regressed when the animals have been permitted to live long enough; in most cases regression has been complete within 35 days after inoculation.

Histologically a 10 day subcutaneous tumor was found to be composed largely of connective tissue cells. These were spindle or polygonal in shape and resembled fibroblasts rather than connective tissue cells. While they were variable in size, most of them were rather large. Their nuclei were round or oval, usually containing clumps of deeply staining chromatin and they possessed an unusual amount of cytoplasm. Mitotic figures were present but not numerous. Occasionally groups of as many as five or six cells whose protoplasm appeared to be continuous were encountered. Intercellular fibrils were abundant and largely collagenous in character. Although the arrangement of the cells was usually loose, some degree of stratification was to be observed. In the lowest layer the long axes of the cells tended to be perpendicular to the base of the tumor and parallel with the nutrient blood vessels. In the layer above, the cells were much more compactly arranged with their long axes generally horizontal. This dense layer was sometimes divided in two by a narrow strip of loose areolar tissue. The upper layer was loosely filled and less definitely arranged than the lowest. In all layers the arrangement was broken by areas of cells which formed whorl-like or otherwise irregular patterns. Scattered among the cells composing the mass of the tumor were small numbers of wandering cells, largely eosinophiles and lymphocytes. At the extreme base of the tumor the lymphocytic infiltration was sometimes marked while just beneath the overlying epithelium were large numbers of eosinophilic and neutrophilic leucocytes. The epithelium did not show the peculiar degenerative changes seen in the original tumor. It was, however, frequently edematous and sometimes showed vesicle formation. The blood supply to the tumor was good and composed largely of small vessels whose walls were usually of only endothelium thickness. In some sections fibroblasts were arranged loosely about blood vessels in such a way as to suggest that they originated from cells in the vascular wall.

In tumors younger than that just described the fibroblastic structure was more loose, the arrangement of the cells more irregular, and mitotic figures were more numerous. In older tumors that were regressing, there was a general, quite marked lymphocytic and leucocytic infiltration, the connective tissue cells stained more faintly and tended to shorten and become round, and the nutrient blood vessels, in many instances, were tightly cuffed with round cells. Small areas of necrosis were also to be seen.

When intramuscular inoculation was successful, large fibrous new growths similar in gross and histological appearance to subcutaneous tumors developed at the site of inoculation, infiltrating the surrounding muscle (Fig. 4).

*Intratesticular Inoculation.*—Rabbits inoculated intratesticularly with a tumor-producing suspension, like those inoculated subcutaneously, showed the first effect on the 3rd to the 5th day. At this time the inoculated testicle was slightly larger and more firm than the normal one, and it continued to increase progressively and rapidly in size and to become more firm. In contrast to the subcutaneous tumor, the testicular tumor continued its growth after the 10th day, not reaching its maximum size until about 20 days after inoculation. Growth became slower, however, after the 10th day. By the 15th to the 20th day, the inoculated testicle seemed to be from 3 to 5 times as large as the uninoculated one when palpated through the scrotum. It was firm, almost hard; and often definite, hard, shotty, projecting nodules could be felt, especially at the upper pole in the region of the epididymis. The scrotum and tunic usually were thickened and edematous and sometimes hard pea-sized, round nodules could be palpated. Not infrequently the inoculated testicle ascended into the abdomen soon after it began its rapid increase in size and remained there.

The typical testicular tumor, 15 to 20 days after inoculation, was found at autopsy to be irregularly lobular in form and 7 to 8 cm. in length and 2 to 3 cm. in its greatest diameter. If it had remained in the scrotum, the tunic, testicle, and scrotum were usually loosely adherent to one another. There were small hard white nodular tumors in the overlying tunic and in the scrotum in addition to the extensive involvement of the testicle. The latter, when removed from the tunic, was found to be mottled purplish white in color, coarsely lobulated, and firm to the touch. The region of the epididymis and cord and almost the entire testicle were composed of what appeared in the gross to be tumor tissue (Fig. 5).

The cut surface of a testicular tumor was similar in appearance to that of a subcutaneous tumor, already described, except that it contained more areas of coagulation necrosis or hemorrhage (Fig. 5). It was white or pinkish white, of a firm rubbery consistency, and appeared to be composed of closely packed and more or less continuous lobulated nodules of varying sizes.

The testicular tumor persisted at its maximum size longer than did the subcutaneous tumor. Large hard nodular testicular tumors were still present 40 days after inoculation, and rabbits autopsied even 2 months after inoculation usually exhibited hard nodular tumors of various sizes on atrophic testicles or in the tunics. Regression, however, could finally be complete, although it was usually accom-

panied by atrophy or marked fibrosis of the involved testicle. Regression was more rapid in cases in which, because of edema or the unusually large size of the tumor, an ischemia of a portion of the overlying scrotum had resulted in a dry gangrene.

Histologically the general types of cells found in testicular tumors were those already described for the subcutaneous tumor. Their arrangement, however, was much more compact and irregular. They formed wide whorls between the seminal tubules, which in most cases had undergone a coagulation necrosis (Fig. 6). In other places they formed more loosely arranged nodules in which were fibroblasts of all sizes and shapes (Fig. 7). Mitotic figures and multinucleated cell masses were more numerous than in the subcutaneous tumors (Fig. 8). Especially to be noted were the masses of young connective tissue cells radiating from blood vessels and forming nodules about them (Fig. 9). Although the blood supply to the testicular tumors was good, it was difficult to find sections without focal areas of necrosis which involved tumor tissue as well as normal structures. This focal necrosis was seen much less frequently in tumors involving only the epididymis and cord. As in subcutaneous tumors, intercellular fibrils, largely collagenous in character, were fairly abundant, small numbers of lymphocytes and eosinophiles were seen, and, near large areas of necrosis, many leucocytes. It cannot be stated definitely whether or not the interstitial cells of Leydig entered into the tumor formation.

The nodular tumors of the tunic presented a peculiar histological picture. In contrast to their firmness on palpation their cells were of extremely loose and irregular arrangement. They were composed largely of fibroblastic cells of moderately compact arrangement at the margins but very loose and widespread in the center. The margins were also frequently densely infiltrated with lymphocytes.

In testicular tumors younger than those just described the fibroblastic invasion of the intertubular spaces was not so extreme, the cells were more irregular and less compact in their arrangement, and more mitotic figures were present. In the gross, testicles bearing younger tumors were purplish red in color, swollen, injected, more edematous and less nodular and less lobulated than those in which the tumor had reached its maximum size. In regressing testicular tumors as in regressing subcutaneous tumors, there was a generalized and extreme lymphocytic infiltration, the connective tissue cells stained more faintly and tended to become rounded, and the nutrient blood vessels were tightly cuffed by lymphocytes.

The possibility that the fibroblastic reaction might be secondary to a preliminary necrosis caused by some agent in the infectious suspensions was eliminated by study of tumors in all stages of development. The first recognizable alteration was hyperplasia of connective tissue and it was never preceded by necrosis. Necrosis, when it did occur, came after the first fibroblastic reaction and appeared to be the result of pressure due to the encroachment of rapidly growing connective



tissue. It was thus the result and not the cause of the fibroblastic proliferation.

Metastases in the neighboring lymph nodes or elsewhere in the body have not been observed in animals inoculated by any route. Occasionally there was an enlargement of the regional lymph nodes draining the area of tumor. Histological preparations of such lymph nodes showed no increased number of connective tissue cells but did show many cells which appeared to be endothelial leucocytes.

### *Experimental Infection of the Wild Cottontail Rabbit*

It has been possible to infect wild cottontail rabbits with the tumor-producing agent that had been passed through a series of laboratory rabbits. The resulting tumors differed in a number of respects, however, from those produced experimentally in laboratory rabbits. One experiment, typical of three that have been conducted, will be outlined here to indicate the differing reactions of wild and domestic rabbits to the tumor-producing agent.

Both a wild rabbit and a laboratory rabbit as control were inoculated subcutaneously with 1 cc. of infectious tumor suspension from a laboratory rabbit of the eighth serial passage. By the 6th day the control had a firm swelling at the site of inoculation measuring 4 x 7 x 1.5 cm. while the wild rabbit had only a pea-sized nodule. On the 10th day the control's tumor reached its greatest size, measuring 5.5 x 8 x 2.5 cm., whereas the nodule on the wild rabbit showed only a slight increase in size. The tumor in the control rabbit regressed more slowly than normally and by the 17th day still measured 6 x 5 x 2.5 cm. and was very firm. The tumor in the wild rabbit at this time was 6 x 4.5 x 1.5 cm. in size, and it reached its maximum size, 7 x 5 x 2 cm., on the 39th day. By this time the control rabbit's tumor had almost completely regressed. On the 77th day after inoculation the wild rabbit was still carrying intact a subcutaneous tumor measuring 6 x 4 x 1.5 cm. Autopsy at this time revealed a tumor identical in gross and histological appearance with those of the original wild rabbit, even to the molluscum contagiosum-like changes in the overlying epidermis. There were no metastases and the animal had shown no general symptoms during the period it was under observation.

### DISCUSSION

The tumors of the original wild rabbit had the general gross and histological appearance of a benign fibroma but were atypical microscopically in that some of the nutrient blood vessels were cuffed with round cells, there was a heavy lymphocytic infiltration of the bases of the tumors, and the overlying epidermis showed degenerative changes

similar to those seen in molluscum contagiosum. Testicular or subcutaneous tumors produced experimentally in laboratory rabbits exhibited a histological picture that was even more suggestive of fibroma, as distinct from granuloma, than was that of the original tumors. In experimentally produced tumors in laboratory rabbits the peculiar degenerative changes seen in the epidermis overlying the tumors in the original wild rabbit did not appear. However, a wild rabbit infected with the tumor-producing agent after eight serial passages through laboratory rabbits exhibited this histological peculiarity. This fact seems to indicate that the difference in the pathological pictures seen in wild and domestic rabbits, as regards the epithelial cells, was one of species difference in reaction to the etiological agent.

Clinically the only manifestation of disease in experimentally infected rabbits was the appearance of tumors at the site of inoculation. There were no general evidences of illness and none died.

The sites of infection were limited to the testicles and subcutaneous tissue. Inoculation of animals intramuscularly or intracutaneously did not regularly lead to local positive evidence of infection. The disease was not contagious, and administration of the tumor-producing agent intraperitoneally, intravenously, or intracerebrally was without apparent effect. It is believed that the inciting agent is effective only when brought into close contact with connective tissue cells, and that it acts upon them directly, causing the formation of a fibroma-like tumor at the site of inoculation.

#### SUMMARY

A tumor-like condition has been observed in a wild cottontail rabbit and has been found to be transmissible to both wild and domestic rabbits but not to guinea pigs, white rats, white mice, or chickens. The clinical picture of the experimentally produced condition and the pathological picture of the original and experimentally produced tumors have been described. The tumor has the general appearance of a fibroma. Methods used in transmitting the condition and satisfactory routes of inoculation have been described and discussed.

#### REFERENCE

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## EXPLANATION OF PLATES

## PLATE 37

FIG. 1. Section of original wild rabbit tumor. The bulbous projections of epidermis are composed of large vacuolated and inclusion-containing epithelial cells. The main mass of the tumor is made up of spindle and polygonal shaped connective tissue cells. Giemsa.  $\times 100$ .

FIG. 2. Subcutaneous tumor on abdomen 11 days after inoculation. The lobular form and sharply demarcated margins of the tumor are characteristic. Dimensions of tumor  $5 \times 6 \times 2.5$  cm.

## PLATE 38

FIG. 3. Cut surface of subcutaneous tumor on abdomen 11 days after inoculation. The tumor is composed of closely packed nodules of glistening white tissue and has the general appearance of a fibroma. Dimensions of tumor  $4 \times 4.5 \times 2$  cm.

FIG. 4. Section of tumor in muscle of the thigh showing young proliferating connective tissue invading spaces between muscle bundles. 11 days after inoculation. Phloxine-methylene blue.  $\times 79$ .

FIG. 5. Intact (on left) and section (on right) of two testicular tumors with a normal rabbit testicle for comparison. The upper testicular tumor was removed 18 days, the lower 16 days after inoculation. In the upper specimen the entire testicle is uniformly replaced by tumor tissue, while in the lower specimen the tumor is limited largely to the region of the epididymis and shows a large area of hemorrhage. Kaiserling-fixed specimens.

## PLATE 39

FIG. 6. Section of testicular tumor showing wide strands of young proliferating connective tissue between degenerating seminiferous tubules. 11 days after inoculation. Giemsa.  $\times 94$ .

FIG. 7. Section of testicular tumor from region of the epididymis showing the general cellular arrangement of an actively growing tumor. The cells are of the young connective tissue type. 11 days after inoculation. Giemsa.  $\times 70$ .

## PLATE 40

FIG. 8. Section of testicular tumor showing comprising the tumor. Mitotic figures and apparently continuous can be seen. In the is the remnant of a degenerating tubule. Hematoxylin-eosin.

FIG. 9. Section of tumor showing proliferating connective tissue cells invading surrounding tissue. Giemsa.  $\times 87$ .

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FIG. 8. Section of testicular tumor showing general appearance of the cells comprising the tumor. Mitotic figures and groups of cells whose protoplasm is apparently continuous can be seen. In the lower left hand portion of the figure is the remnant of a degenerated seminiferous tubule. 11 days after inoculation. Hematoxylin-eosin.  $\times 306$ .

FIG. 9. Section of testicular tumor showing whorl-like arrangement of young proliferating connective tissue about nutrient blood vessels. Connective tissue cells invading surrounding necrotic tissue at margins. 11 days after inoculation. Giemsa.  $\times 87$ .



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FIG. 2. Subcutaneous tumor on abdomen 11 days after inoculation. The lobular form and sharply demarcated margins of the tumor are characteristic. Dimensions of tumor 5 x 6 x 2.5 cm.

## PLATE 38

FIG. 3. Cut surface of subcutaneous tumor on abdomen 11 days after inoculation. The tumor is composed of closely packed nodules of glistening white tissue and has the general appearance of a fibroma. Dimensions of tumor 4 x 4.5 x 2 cm.

FIG. 4. Section of tumor in muscle of the thigh showing young proliferating connective tissue invading spaces between muscle bundles. 11 days after inoculation. Phloxine-methylene blue.  $\times 79$ .

FIG. 5. Intact (on left) and section (on right) of two testicular tumors with a normal rabbit testicle for comparison. The upper testicular tumor was removed 18 days, the lower 16 days after inoculation. In the upper specimen the entire testicle is uniformly replaced by tumor tissue, while in the lower specimen the tumor is limited largely to the region of the epididymis and shows a large area of hemorrhage. Kaiserling-fixed specimens.

## PLATE 39

FIG. 6. Section of testicular tumor showing wide strands of young proliferating connective tissue between degenerating seminiferous tubules. 11 days after inoculation. Giemsa.  $\times 94$ .

FIG. 7. Section of testicular tumor from region of the epididymis showing the general cellular arrangement of an actively growing tumor. The cells are of the young connective tissue type. 11 days after inoculation. Giemsa.  $\times 70$ .

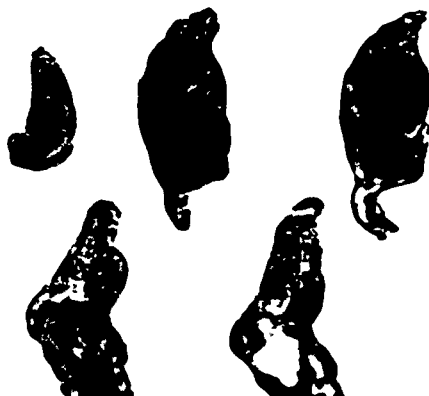
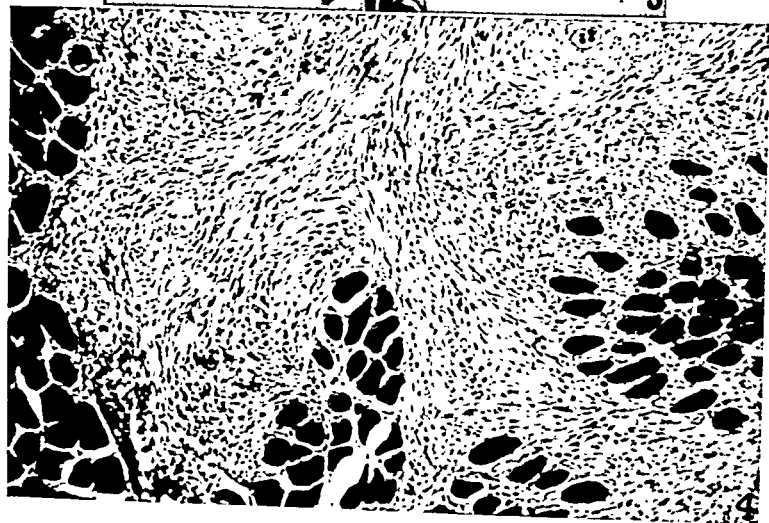
## PLATE 40

FIG. 8. Section of testicular tumor showing general appearance of the cells comprising the tumor. Mitotic figures and groups of cells whose protoplasm is apparently continuous can be seen. In the lower left hand portion of the figure is the remnant of a degenerated seminiferous tubule. 11 days after inoculation. Hematoxylin-eosin.  $\times 306$ .

FIG. 9. Section of testicular tumor showing whorl-like arrangement of young proliferating connective tissue about nutrient blood vessels. Connective tissue cells invading surrounding necrotic tissue at margins. 11 days after inoculation. Giemsa.  $\times 87$ .













A FILTRABLE VIRUS CAUSING A TUMOR-LIKE CONDITION IN RABBITS AND ITS RELATIONSHIP TO VIRUS MYXOMATOSUM

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In a preceding paper (1) the clinical and pathological characteristics of a tumor-like condition of rabbits were described. The purpose of the present paper is to report experiments indicating the nature of the etiological agent and to point out its possible relationship to the virus of infectious myxoma. As in the preceding paper, the term "tumor" is used in its broadest sense, to indicate a local swelling consisting of a mass of new tissue.

*Viability of the Tumor-Inducing Agent in Glycerol*

Tumor tissue from domestic rabbits stored in 50 per cent glycerol at refrigerator temperature for as long as 86 days lost very little, if any, of its original potency as judged by the size and character of the tumors it produced in domestic rabbits. Tissue stored for a longer period has not been tested for infectivity.

*Etiology*

Cultures of tumor tissue on various media, including some suitable for the growth of tubercle bacilli, failed to reveal any significant organism.

The resistance of the etiological factor to glycerol, together with the finding of inclusion-like bodies in the cytoplasm of epithelial cells overlying the original wild rabbit tumor (1), suggested that the causative agent might be a filtrable virus. Attempts were made to transmit the condition to domestic rabbits by means of sterile filtrates of infectious suspensions.



Tumor tissue to be used as a source of infection in the filtration experiments was minced with sterile scissors, ground in a mortar with sterile sand, and suspended in infusion broth (pH 7.3) sufficient to make a 5 per cent suspension. Suspensions thus prepared were cleared of gross particles by centrifugation and the supernatant fluid was filtered through paper. 1 cc. of 24 hour broth cultures of *B. prodigiosus* was added to each 15 to 20 cc. of the paper filtrates and they were then filtered through Berkefeld candles. The resulting filtrates were tested for sterility in amounts of 1.5 to 3 cc. All those recorded were bacteriologically sterile.

The results of the filtration experiments are summarized in Table I.

The testicular tumors produced by filtrates were as large, persistent, and characteristic as those in the control animals which had received unfiltered suspensions. The subcutaneous tumors, on the other hand, were usually smaller, did not last so long, and were less compact than those in control animals. In some of the filtrate-infected animals the subcutaneous reaction consisted in the formation of groups of small, firm, shotty nodules lying in a softer subcutaneous swelling. These regressed very rapidly and without sloughing. Testicular tumors produced by filtrates, however, when used to infect further series of rabbits subcutaneously, caused typical firm, compact tumor formation. The period between the time of inoculation and the first evidence of tumor formation was approximately 2 days longer in animals receiving Berkefeld V and N filtrates than in those receiving the unfiltered suspensions. Only one of the two attempts to pass the tumor-producing agent through Berkefeld W filters was successful, and the incubation period in the rabbit receiving this filtrate was 10 days in contrast to 4 days in the case of its control receiving unfiltered suspension.

#### *Immunity Conferred by Experimental Infection*

Domestic rabbits in which either a subcutaneous or testicular tumor had been produced and allowed to regress were completely resistant to reinfection by either route. This immunity became evident before the tumor had completely regressed. Actual tumor formation was necessary for the production of immunity, since an amount of agent that failed to produce a tumor conferred no demonstrable immunity.

The neutralization experiments summarized in Table II show that sera from rabbits whose tumors had regressed were capable of neutral-

TABLE I  
Filtration Experiments

Filter		Material filtered (approximately 5% suspension of testicle and subcutaneous tumor Rabbit No.)	Time of filtration	Amount of filtrate	Maximum nega- tive pressure	Rabbit inoculated, No.	Amount of filtrate injected and route		Result, tumor formation at site of inoculation
Type	No.								
Berkefeld V	13	248*	1.5	30	62	268	1	i.t.†	Positive
"	N	8 268 and 269	4	6	62	271	1	"	"
"	"	18 281	12	42	62	286	1	s.c.	" but slight
							1	i.t.	"
						287	1	s.c.	" but slight
							1	i.t.	"
						289	1	s.c.	" but slight
							1	i.t.	"
						290	1	s.c.	"
							1	i.t.	"
						292	1	s.c.	" but slight
							1	i.t.	"
						293	1	s.c.	" but slight
							1	i.t.	"
						294	1	s.c.	" but slight
							1	i.t.	"
Berkefeld V	13	300	10	33	62	304	3	s.c.	" but slight
							1	i.t.	"
						305	3	s.c.	"
							1	i.t.	"
						306	3	s.c.	"
							1	i.t.	"
						307	3	s.c.	"
							1	i.t.	"
						308	3	s.c.	"
							1	i.t.	"
						309	3	s.c.	" but slight
							1	i.t.	"
Berkefeld W	9	369 and 409	15	10	62	422	2	s.c.	Negative
							1	i.t.	"
Berkefeld W	11		15	25	62	423	2	s.c.	"
							1	i.t.	Positive

\* The numbers in this and the subsequent tables are taken from laboratory records and bear no relation to the actual number of animals used in these experiments.

† i.t. = intratesticularly. s.c. = subcutaneously.

TABLE II  
*Neutralization Tests with Serum from Recovered Rabbits*

Source of serum	Source of tumor-producing agent	Rabbit inoculated, No.	Amount of mixture of infectious suspension and serum injected and route of injection*	Result, tumor formation at site of inoculation
Recovered Rabbit 234	Supernatant fluid suspension testicular tumors Rabbits 279 and 280	298	"	Negative
Physiological saline		299	2.0 s.c.†	Positive; long latent period
		(control)	0.6 i.t.	"
			2.0 s.c.	"
Recovered Rabbit 266	Supernatant fluid suspension subcutaneous and testicular tumors Rabbit 307	312	0.6 i.t.	"
"		313	2.0 s.c.	Negative
"		314	0.2 i.t.	Positive but slight
"		315	2.0 s.c.	Doubtful small tumor
"		316	0.2 i.t.	Positive; long latent period
"		311	2.0 s.c.	Negative
Normal		(control)	0.2 i.t.	Positive but slight
			2.0 s.c.	"
Recovered " 263	Supernatant fluid suspension testicular tumor Rabbit 380	387	2.0 s.c.	Negative
Normal " 14		392	0.4 i.t.	"
		(control)	2.0 s.c.	Positive
			0.4 i.t.	"

\* The mixtures injected were comprised of equal parts of infectious suspension and convalescent serum.

† s.c. = subcutaneously; i.t. = intratesticularly.

izing the tumor-producing agent completely in tests by subcutaneous inoculation and partially or completely in tests by testicular inoculation.

In this and other neutralization tests described in this paper the mixtures of serum and infectious suspension were incubated at 37°C. for 1 hour and then stored for 2 hours in the refrigerator before use.

TABLE III

*Period of Persistence of Tumor-Producing Agent in Tumors*

Source of infectious material, Rabbit No.	Length of time after inoculation that material was taken	Material used for inoculation, suspension of	Rabbit inoculated, No.	Route of inoculation and result, tumor formation at site of inoculation
307	7	Subcutaneous tumor	317	s.c.,* positive
		Testicular "	318	i.t., " s.c., " i.t., "
305	14	Subcutaneous "	335	s.c., negative
		Testicular "	336	i.t., positive s.c., " i.t., "
311	21	Subcutaneous "	346	s.c., negative
		Testicular "	347	i.t., positive s.c., " but slight i.t., "
318	35	Testicular "	359	s.c., negative i.t., positive but slight

\* s.c. = subcutaneously. i.t. = intratesticularly.

The time necessary for the appearance of antibodies effective against the tumor-producing agent in the blood serum of tumor-bearing domestic rabbits has not been determined. They were, however, present in the serum of 1 animal (Rabbit 305, Table III) 14 days after inoculation. It should be noted that at this time the rabbit had large subcutaneous and testicular tumors that were shown to contain the tumor-producing agent.

*Persistence of the Tumor-Producing Agent in Experimental Tumors*

Experiments to determine the most favorable time for transmitting the tumor in series have yielded the results recorded in Table III. All rabbits furnishing infectious material in these experiments were inoculated both subcutaneously and intratesticularly and developed typical tumors at both sites.

These results are only sufficient to suggest that the tumor-producing agent disappears or becomes attenuated before the regression of the tumor and that the threshold of natural resistance to infection is higher by the subcutaneous than by the testicular route. The agent persisted in an experimentally produced subcutaneous tumor in a wild cottontail rabbit for at least 77 days after inoculation and material taken from this tumor was still capable of inducing tumor formation when administered either subcutaneously or intratesticularly to domestic rabbits. This relatively long persistence of the tumor-producing agent in a wild rabbit tumor is in marked contrast to its early disappearance from domestic rabbit tumors.

*Failure to Demonstrate the Tumor-Producing Agent in the Blood*

Blood from infected domestic rabbits 2, 7, and 8 days after inoculation did not induce tumor formation when administered subcutaneously, intracutaneously, or intratesticularly. Animals so tested were found subsequently to be fully susceptible to material known to contain the tumor-producing agent.

*Fate of Tumor Grafts*

While the implantation of bits of tumor tissue into the testicle or under the skin of domestic rabbits with a large aspirating needle, after the method usually employed in tumor grafting, frequently, although not invariably, gave rise to local tumor formation, histological study of such tumors has been inconclusive in showing that multiplication of cells in the engrafted bit of tumor ever took place. The impression gained from the study of sections of such graft-produced tumors was that they arose as a result of stimulation of surrounding connective tissue by an agent present in the graft. As yet no sufficient experiments have been conducted to establish definitely the fate of grafts. It has been possible, however, to produce tumors by the implantation of grafts from the tumor of a wild cottontail rabbit in domestic rabbits

and, conversely, in a wild cottontail rabbit by the implantation of a graft from the tumor of a domestic rabbit. In view of the generally accepted evidence regarding the impossibility of successfully transferring the tissues of one species to another, the introduction of the tumor-producing agent with the graft seems more probably the cause of ensuing tumor formation than the proliferation of cells introduced with the graft.

### *Relation of the Tumor-Producing Agent to Virus III<sup>1</sup>*

Five male domestic rabbits immunized to Virus III by testicular and cutaneous infection were found, when tested 1 to 2 months later, to be fully susceptible to the tumor-producing agent administered subcutaneously or intratesticularly. This seemed to eliminate the possibility that Virus III, which might have become associated with the tumor-producing agent by rapid serial testicular passage through rabbits, contributed in any way to the tumor-like condition.

### *Relation of the Tumor-Like Condition to Infectious Myxoma<sup>1</sup>*

The strain of myxoma virus used in the experiments to be described here was that used by both Hobbs (2) and Rivers (3) and was originally obtained from Dr. Arthur Moses of the Oswaldo Cruz Institute in Brazil. Since, in recent works, Hobbs (2) and Rivers (3) have very fully described the characteristics of this strain of myxoma virus and the clinical and pathological manifestations of the disease it produces under experimental conditions, only the similarities and differences between infectious myxoma and the tumor-like condition under discussion will be summarized.

Since Moses (5) has found the wild rabbit of Brazil insusceptible to experimental infection with *Virus myxomatosum* except in rare instances, and Hobbs (4) has been unable to infect our native wild cottontail rabbits with the virus, there was no expectation that the condition observed in the original wild rabbit was infectious myxoma. The character of the experimentally produced tumor-like condition in laboratory rabbits spoke strongly against the view that we were dealing with infectious myxoma. The salient characteristics in

<sup>1</sup> The Virus III and *Virus myxomatosum* used in these experiments were kindly supplied by Dr. T. M. Rivers of The Rockefeller Institute for Medical Research.

which the two conditions differed may be briefly indicated here. Infectious myxoma is almost uniformly fatal for rabbits and is characterized not only by a local swelling at the site of inoculation but by edematous swellings about the eyes, ears, nose, mouth, external genitalia, and other parts of the body; while the tumor-like condition is in our experience never fatal, its only evidences of pathology are at the site of inoculation, and it produces no general symptoms. The virus of myxoma is infectious by any route and the resulting disease is highly contagious, whereas the tumor-producing agent is effective only when it can be brought into intimate contact with connective tissue, and the resulting disease is not contagious. The virus of infectious myxoma is found regularly in the blood stream while the tumor-producing agent has not been demonstrated there. The swelling produced at the site of inoculation with myxoma virus is of a diffuse character with an indefinite margin and it has the gross and histological appearance of an edematous inflammatory cellulitis accompanied by connective tissue proliferation. The tumor, on the other hand, arising at the site of inoculation with tumor-producing agent is firm and well circumscribed; it has the gross and histological appearance of a fibroma. One should add that portions of myxoma lesions can be found which bear a closer resemblance to a neoplastic process than they do to an inflammatory one. In the epithelial cells overlying a myxoma swelling, eosinophilic cytoplasmic inclusions can regularly be seen. These are not found in the epithelium overlying the tumor nodules produced in laboratory rabbits although the epidermis may be edematous and vesiculated.

In spite of these differences, very early in the present work two characteristics of the tumor-like condition suggested a possible relationship to infectious myxoma. The first was the spongy consistency of the local swelling in its early stage before it had developed its characteristic firm tumor-like form. The second was the finding of cytoplasmic eosinophilic inclusions very similar to those seen in infectious myxoma in the epithelium overlying the original wild rabbit tumor and in that overlying an experimentally produced tumor in a wild rabbit. The following experiments were undertaken in order to explore this possible relationship.

*Resistance to Infectious Myxoma Exhibited by Rabbits Recovering from the Tumor*

Domestic rabbits in which actively growing subcutaneous or testicular tumors had been allowed to regress either partially or completely were tested for susceptibility to infectious myxoma. Of these, 8 were inoculated subcutaneously with a very large dose of myxoma virus, while 7 received, in the same way, a somewhat smaller dose. The results are recorded in Table IV.

As shown in this table, only 1 of the 15 rabbits died of infectious myxoma, 2 proved completely resistant, and the others, although they exhibited varying degrees of illness, all recovered. This result, striking in view of the practically uniform fatality of the disease as observed by other investigators, indicated that rabbits, in which tumors had regressed, had been rendered more than normally resistant to infection with the virus of myxoma.

*Attempts to Neutralize the Virus of Infectious Myxoma with Serum of Rabbits Recovered from Tumor*

In an attempt to obtain information concerning the nature of the increased resistance to infectious myxoma exhibited by rabbits recovered from tumor, the sera of such animals were tested for antibodies effective against the myxoma virus. These sera had previously been shown to contain antibodies effective against the tumor-producing agent. The results of such cross-neutralization tests were completely negative, as shown in Table V.

*Neutralization of the Virus of Infectious Myxoma with Serum from Tumor-Recovered Rabbits That Had Been Subsequently Inoculated with Myxoma Virus*

While the sera from rabbits recovered from tumor failed to neutralize myxoma virus, these same rabbits when subsequently infected with myxoma underwent a mild and abortive type of disease after which their sera neutralized the virus of infectious myxoma, as shown by Table VI.



## VIRUS CAUSING A TUMOR-LIKE CONDITION

TABLE IV  
*Resistance of Tumor-Immune Rabbits to Infectious Myxoma*

Rabbit No.	Site of tumor	Condition of tumor at time of myxoma inoculation	Dosage of myxoma virus subcutaneously	Clinical picture	Result
226	Both testicles	Almost complete regression	1 cc. of the supernatant of a 10% suspension of subcutaneous swelling and testicle of Rabbit 284	Severe illness	Recovered
233	Subcutis	Complete regression		Moderate illness	"
234	"	"		Mild abortive illness	"
242	Both testicles	"		Abortive very mild illness	"
243	"	"		Severe illness	"
265	"	Regressing	0.5 cc. of a 1:50 dilution of the supernatant of a 5% suspension of subcutaneous swelling and testicle of Rabbit 310	Abortive mild illness	"
267	Subcutis	Complete regression		Moderate illness	"
271	Both testicles	Regressing		Local subcutaneous swelling	No illness
297		Control		Typical myxoma	Died on 7th day
251	Both testicles	Regressing		Completely negative	No illness
263	Subcutis and testicle	Almost complete regression		Local subcutaneous swelling	"
264	Both testicles	"		Typical myxoma	Died on 12th day
266	"	"		Local subcutaneous swelling	No illness
283	Subcutis	Regressing		"	"
320		Almost complete regression	1 cc. of a 1:50 dilution of the supernatant of a 5% suspension of subcutaneous swelling and testicle of Rabbit 421	Typical myxoma	Died on 9th day
321		Control		"	" 10th "
416	Both testicles	Beginning regression		Local subcutaneous swelling	No illness
417	Subcutis and both testicles	"		Completely negative	"
426		Control		Typical myxoma	Died on 7th day

TABLE V  
*Attempts to Neutralize the Virus of Infectious Myxoma with Serum from Rabbits Recovered from the Tumor*

Source and concentration of myxoma virus	Rabbit No.	Amount of virus subcutaneously cc.	Serum		Result
			Source	Amount cc.	
Supernatant of a 10% suspension of subcutaneous swelling and testicle of Rabbit 284	296	1	Recovered Rabbit 234	1	Died in 9 days
	297 (control)	1	Normal	1	" " 7 "
Supernatant of a 5% suspension of subcutaneous swelling and testicle of Rabbit 310	295	0.5, 1:50 dilution	Recovered	0.5	" " 11 "
	322	0.5, 1:50 "	"	0.5	" " 7 "
	323	0.5, 1:50 "	"	0.5	" " 11 "
	324	0.5, 1:50 "	"	0.5	" " 12 "
	325	0.5, 1:50 "	"	0.5	" " 10 "
	320	0.5, 1:50 "	Normal	0.5	" " 9 "
	(control)				
	321 (control)	0.5, 1:50 "	"	0.5	" " 10 "
Supernatant of a 5% suspension of subcutaneous swelling Rabbit 344	367	0.5, 1:25 "	Recovered	1.5	" " 13 "
	368	0.5, 1:25 "	"	1.5	" " 15 "
	365 (control)	0.5, 1:25 "	Normal	1.5	" " 12 "

TABLE VI  
*Neutralization of the Virus of Infectious Myxoma with Serum from Tumor-Recovered Rabbits Subsequently Inoculated with Myxoma Virus*

Source and concentration of myxoma virus	Rabbit No.	Amount of virus subcutaneously	Serum		Result
			Source	Amount	
Supernatant of a 5% suspension of subcutaneous swelling and testicle of Rabbit 310	338	cc. 0.5, 1:50 dilution	Rabbit 234 after recovery from myxoma	cc. 0.5	Moderate illness; recovered
	337 (control)	0.5, 1:50 "	Normal Rabbit 14	0.5	Died in 12 days
	364	0.5, 1:25 "	Rabbit 234 after recovery from myxoma	1.5	No illness
Supernatant of a 5% suspension of subcutaneous swelling of Rabbit 344	366	0.5, 1:25 "	Rabbit 266 after recovery from myxoma	1.5	"
	365 (control)	0.5, 1:25 "	Normal Rabbit 14	1.5	"
				1.5	Died in 12 days

*Inoculation of Wild Cottontail Rabbits with the Virus of Infectious  
Myxoma and Its Effect on Their Susceptibility to the Tumor-  
Producing Agent*

Hobbs (4) has stated that in his experience the native wild cottontail rabbit is not susceptible to infectious myxoma. Two out of three of our trials to infect wild cottontail rabbits also yielded apparently negative results. In the third attempt the very large dosage of 2 cc of a 5 per cent suspension of virus-containing tissue administered subcutaneously resulted, after 16 days, in a transitory thickening of the epidermis and subcutaneous tissue in the region of the site of inoculation. No general symptoms or characteristic swellings of the eyelids, nose, and external genitalia were observed. The clinical picture of this one wild rabbit was thus at wide variance with both infectious myxoma as produced in laboratory rabbits and the tumor-like condition produced in wild and laboratory rabbits. These few experiments with wild cottontail rabbits tend to confirm Hobbs' observation that this species does not develop clinically recognizable infectious myxoma as a result of inoculation with myxoma virus.

However, in spite of the apparently negative reaction of 2 of 3 animals to the virus of infectious myxoma, they, as well as the one that had developed a doubtful myxoma lesion, were subsequently found to be resistant to the tumor-like condition. Blood serum obtained a month after infection, from the wild rabbit that had shown a doubtfully positive reaction to the virus of infectious myxoma, exhibited only slight inhibitory action on both the tumor-producing agent and the myxoma virus in neutralization tests.

*Neutralization of the Tumor-Producing Agent by Serum of a Rabbit Convalescent from Infectious Myxoma*

While a number of domestic rabbits have been rendered immune to myxoma by preliminary infection with the tumor-producing agent and while, after subsequent inoculation with myxoma virus, the serum of such animals have proved capable of neutralizing that virus (Table VI), they were not satisfactory to use in cross-neutralization experiments because of the earlier tumor infection. Consequently experiments were initiated in order to secure a combination of ser-

TABLE VII  
*Neutralization of Tumor-Producing Agent and Myxoma Virus by the Serum of a Rabbit Recovered from Myxoma*

Type and concentration of infectious agent	Rabbit No.	Amount of infectious suspension administered and route		Serum		Result
		cc.		Source	Amount	
Supernatant of a 5% suspension of myxomatous material from Rabbits 344 and 365	390	0.5, 1:25	dilution, s.c.*	Myxoma-convalescent Rabbit 338	1.5	No illness
	388 (control)	0.5, 1:25	"	Normal Rabbit 14	1.5	Died in 18 days
Supernatant of a 5% suspension of testicular tumor from Rabbit 380	385	1	"	Myxoma-convalescent Rabbit 338	1	No tumor
	392 (control)	0.2	i.t.	Myxoma-convalescent Rabbit 338	0.2	Scant tumor growth
Supernatant of a 5% suspension of testicular tumor from Rabbit 392	396 (control)	1	s.c.	Normal Rabbit 14	1	Typical tumor growth
		0.2	i.t.	" 14	0.2	" "
	394 (control)	1	s.c.	Myxoma-convalescent Rabbit 338	1	No tumor
		0.2	i.t.	Myxoma-convalescent Rabbit 338	0.2	Scant tumor growth
		1	s.c.	Normal Rabbit 14	1	Typical tumor growth
		0.2	i.t.	" 14	0.2	" "

\* s.c. = subcutaneously. i.t. = intratesticularly.

and myxoma virus such that, while preventing a fatal myxoma illness, it would still render the animal immune. Most of the mixtures were so balanced that either there was no evidence of neutralization and the animals died much like their controls, or the neutralization was complete and neither illness nor immunity resulted. One animal (Rabbit 338), however, either because the partially neutralized virus was ideal for immunization, or because of a natural partial resistance to infectious myxoma, developed a non-fatal attack of the disease and recovered completely. Serum from this rabbit was then tested for neutralizing properties against the myxoma virus and the tumor-producing agent. The results of these experiments are recorded in Table VII.

As shown in Table VII, the serum from Rabbit 338 contained antibodies effective against both the myxoma virus and the tumor-producing agent. This animal was subsequently inoculated subcutaneously with tumor-producing agent and found to be resistant to infection.

#### DISCUSSION

The properties of the tumor-producing agent described in this and the preceding paper (1) are all of the group generally considered characteristic of a filtrable virus. The failure to cultivate any organisms from the tumors or to see them in stained sections, together with the tumor-producing agent's resistance to glycerol, its ready filtrability, the type of immunity it induces, its relative host specificity, its production of cytoplasmic inclusions in the epithelial cells of one of its susceptible hosts, and its apparent tropism for one type of tissue, considered collectively, suffice to place it in the general group of filtrable viruses. Any attempt to classify it in any other way would seem artificial.

The classification of the pathological product of the action of the virus on the host is more difficult. The original tumor seen in the wild rabbit, and, in fact, those produced experimentally in wild rabbits, could, from the pathological picture, especially that revealed by the microscope, be classed as either granulomata or fibromata. However, the gross and histological pictures of the tumor as produced in laboratory rabbits, whether by intratesticular, subcutaneous, or

intramuscular inoculation, resemble those of a fibroblastic neoplasm. The extremely rapid growth of the tumor during the first 10 or 15 days after inoculation, followed later by retrogression, is not usual with transplanted neoplasms. It should be remembered, however, that this is the event in an alien species, and that mouse tumors will grow for a time in rats before retrogressing. Tumors produced in wild rabbits appear to persist unchanged indefinitely, to judge from the few available instances. The production of the tumor by material stored for long periods of time in glycerol distinguishes it from mammalian neoplasms, as also its production by cell-free filtrates. However, these features taken together fail to eliminate the possibility that the tumor is of neoplastic character, for the Rous sarcomas of chickens can be produced by cell-free filtrates of tumor tissue and by glycerolated material. As yet sufficient experiments on the fate of engrafted material have not been conducted to enable one to say whether the introduction of such material involves an actual transplantation of the growth. There can be no doubt that the tumors caused by the injection of filtrates arise as a result of the local action of the virus on the cells of the injected animal. The fibroma-like new growths are the product of this reaction. There is evidence (1) that the effectiveness of the virus in the production of tumors depends upon its being brought into intimate contact with connective tissue cells and that it bears a relationship to connective tissue similar to that borne by neurotropic viruses for the central nervous system or dermatotropic viruses for the skin. From the data at hand the tumor can better be considered as the local hyperplastic connective tissue reaction to the virus than as a true neoplastic process.

When the tumor-like condition and infectious myxoma were compared, utilizing cross-immunity phenomena to determine relationship, surprising results were encountered in view of the marked clinical and pathological differences already discussed. It was found, in fourteen out of fifteen instances, that rabbits in which tumors had regressed or were regressing exhibited marked resistance to infection with the myxoma virus. In only 2 out of 14 animals was this resistance of such a degree as to be classified as complete. In the remaining 12 rabbits myxoma developed in degrees of severity varying from a local lesion at the site of inoculation to a grave illness. Blood serum

from tumor-convalescent rabbits, however, while capable of neutralizing the tumor virus, did not protect against *Virus myxomatosum*. Even the serum from Rabbit 251 (Tables IV and V) was devoid of neutralizing properties against myxoma virus although this animal had proved completely resistant to infectious myxoma on test inoculation. These experiments indicate that the failure of the virus of infectious myxoma to produce a fatal illness in tumor-convalescent rabbits may be explainable on the basis of an acquired resistance, conceivably of fixed tissue type. This view was strengthened by the finding that a rabbit (No. 266), whose serum drawn after recovery from the tumor failed to neutralize myxoma virus (Table V), after subsequent infection with myxoma virus, which resulted in only a local swelling, yielded a serum capable of neutralizing myxoma virus (Table VI). The fact that myxoma virus, which in a tumor-convalescent rabbit produced only a local lesion, was nevertheless capable of establishing demonstrable humoral antiviral bodies, while tumor virus, also causing a local lesion, developed no demonstrable humoral antibodies but did establish a resistant state in the host, seems significant. Two possible interpretations of these observations are apparent. The first and probably least likely is that a fine line of distinction may be drawn between the *resistance* to infectious myxoma generated in a rabbit by its previous infection with tumor virus and the *immunity* it acquires when it is subsequently inoculated with myxoma virus. Under such an interpretation the two viruses could be considered as antigenically distinct from one another. The second and simpler explanation for the immunological differences is that they are quantitative only, not qualitative, and that the tumor virus establishes a lower grade of immunity against *Virus myxomatosum* merely by virtue of being a poorer antigen than *Virus myxomatosum*. Under this second interpretation the two viruses would be thought of as antigenically identical, or closely related to one another. The tumor virus would then be considered merely as a strain of *Virus myxomatosum*, atypical, however, in its ability to regularly infect wild rabbits, in its inability to produce the classically rapidly fatal infectious myxoma in domestic rabbits, and in its failure to generate in domestic rabbits demonstrable virucidal antibodies effective against *Virus myxomatosum*. The tumor virus, considered as a strain of *Virus*



*myxomatosum*, would further differ from our classical conception of that virus by being incapable of passage from rabbit to rabbit by contact, by not invading the blood stream, by not establishing a generalized infection with widespread pathological alterations, by failing completely to infect domestic rabbits when administered intravenously or intraperitoneally, by having an apparently specific infective affinity for connective tissue, and by producing at the site of inoculation a tumor histologically at wide variance with the local lesion produced by *Virus myxomatosum*.

Wild cottontail rabbits inoculated with *Virus myxomatosum* failed, with one possible exception, to show clinical evidence of myxoma, but in spite of this became subsequently resistant to infection with tumor virus. This acquired resistance too was not accompanied by the appearance in the blood serum of antibodies neutralizing either myxoma or tumor virus.

The cross-immunity developed by a rabbit convalescent from myxoma has also been considered. Hobbs (4) demonstrated that serum from a rabbit convalescent from infectious myxoma would neutralize the myxoma virus and Fisk and Kessel (6) found that rabbits convalescent from infectious myxoma were immune to subsequent infection by the virus. We were desirous of determining whether rabbits convalescent from infectious myxoma would show resistance or immunity to the tumor virus. Only one animal not previously infected with tumor virus has so far survived an attack of myxomatosis. This rabbit was shown thereafter to be resistant to infection with tumor virus and its serum not only neutralized myxoma virus completely but also possessed virucidal properties for the tumor virus.

The relationship of the tumor-producing virus to the myxoma virus is not yet clear. The two diseases appear at present as separate clinical entities produced by viruses that seem immunologically to be related. The recent work of Andrewes (7) on the immunological relationships of fowl tumors with different histological structure and that of Murphy and his coworkers (8, 9) on a tumor-inhibiting substance effective even against tumors of an alien host may point the way to an explanation of the peculiar relationship existing between the two. Another possibility is that their relationship is like that of

vaccine virus and the virus of variola. One might suppose that the two viruses were originally identical or at least had a common ancestor, and that the tumor virus was developed from the myxoma virus by prolonged passage through wild rabbits just as the virus of variola, after passage in series through calves, becomes permanently vaccine virus, or so it is supposed.

Rivers (3), in discussing similarities between the myxoma of rabbits and the Rous sarcoma of chickens, has said, "If, as some believe, the Rous sarcoma appears to be more closely related to true neoplasms than to diseases induced by highly contagious agents, then the myxoma, upon further study, may serve to bridge the gap between the Rous tumor and other virus maladies. . . ." The tumor-like condition of rabbits discussed in this and the preceding paper probably has a place somewhere between infectious myxoma and the Rous sarcoma in this general arrangement of virus diseases suggested by Rivers, thus bridging still further the gap between the Rous tumor and other virus maladies.

The description by von Dungern and Coca (10) of a transmissible spindle cell fibrosarcoma in wild hares, which could be transplanted to laboratory rabbits, while presenting interesting points of resemblance to the disease under discussion here, was not accompanied by sufficient data on the nature of the inciting agent for a close comparison.

#### SUMMARY

The properties of the agent causing a tumor-like condition in rabbits have been tested experimentally and the conclusion reached that it is a filtrable virus. While the tumor-like condition and infectious myxoma differ markedly in their clinical and pathological pictures, they have been found to be related immunologically. The relationships of the tumor-producing virus to the virus of infectious myxoma, and of the tumor-like condition to malignant neoplasms have been discussed.

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AMMONIUM CHLORIDE DECALCIFICATION, AS MODIFIED  
BY CALCIUM INTAKE: THE RELATION BETWEEN  
GENERALIZED OSTEOPOROSIS AND OTITIS  
FIBROSA

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PLATES 41 AND 42

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Experimental hyperparathyroidism in young and old animals on high and low calcium intakes results in bone decalcification and secondary fibrosis leading to the production of generalized otitis fibrosa (1-6). We conceived the mechanism of these changes as follows:

"It may well be that the immediate antecedent of experimental otitis fibrosa is a condition, perhaps related to the disturbed acid-base equilibrium, which may also be caused by other agents than parathormone. However, it seems essential that the action be continuous, and that the condition which it causes be maintained for long periods without endangering the life of the experimental animal, or a certain minimum of well-being. Parathormone is specific in the sense that it satisfies these requirements" (1).

We recognized, in view of the known effects of parathormone upon the mineral metabolism, that other means of similarly affecting mineral metabolism might produce similar end-results. It was deemed desirable to undertake a quantitative study of the question of experimental bone decalcification, using ammonium chloride to bring about generalized osteoporosis and possibly otitis fibrosa.

Ammonium chloride has long been known to be an effective agent in producing acidosis, as in its metabolism urea is synthesized from the ammonium radicle and hydrochloric acid is liberated. Ammonium chloride may be administered in considerable quantities for a long time. Haldane demonstrated an increased excretion of bases and phosphates in the experimental acidosis thus produced (7). The

calcium reserves in the bone may be expected to take a considerable part in the neutralization of the acid, and an increased excretion of calcium and phosphorus, especially through the kidneys, has been confirmed experimentally (8).

Increases of serum phosphatase were demonstrated in the course of this study, which were roughly parallel to the degree of bone decalcification (9).

### *Methods*

Four litters of dogs were used, 3, 6, 8, and 18 months old, respectively, at the beginning of the experiment. At the outset the 3 months old puppies averaged about 3 kilos, the 6 months about 8 kilos, the 8 months old dogs about 10 kilos, and the 18 months old dogs about 17 kilos. Each group contained one or more control animals which did not receive ammonium chloride. Two types of control were used, those maintained on a calcium-adequate diet and those on a calcium-inadequate diet. The experiment was continued for 11 weeks.

*Diet.*—The diet consisted of fresh ground lean horse meat, mixed with 2 per cent cod liver oil and 5 per cent canned tomatoes. This constituted the calcium-poor diet. It was adequate in other respects. The animals receiving the calcium-adequate diet were given a supplement consisting of 2.5 gm. of bone meal and 2.5 gm. calcium lactate (equivalent to about 1 gm. calcium) per kilo of meat mixture. On this diet the control animals grew well and were in excellent condition. The food intake, though measured daily, was given practically *ad libitum*. The animals consumed from 0.5 to 2.5 kilos per day, depending on their size. The youngest dogs on the low calcium diet with ammonium chloride supplement suffered loss of appetite towards the end of the experiment and their food consumption decreased to about 0.5 kilos per day.

*Ammonium Chloride Administration.*—Ammonium chloride was administered at first in the form of a 1 per cent solution by stomach tube, and later in 1½ per cent and 2 per cent solutions, once a day at the beginning of the experiment, and twice a day when the volume administered would otherwise have been too large to be given at one time. At the beginning of the experiment the amount of ammonium chloride was about 0.08 gm. per kilo of body weight per day, for all the groups. It was increased relatively rapidly in the 18 months old dogs to a final daily dose of 1 gm. per kilo body weight, in the 8 months old dogs to 0.8 gm. per kilo, in the 6 months old dogs to 0.7 gm. per kilo, and in the 3 months old dogs to 0.4 gm. per kilo. The final dose was continued for 17 days before the conclusion of the experiment.

### *Course of the Experiments*

The control animals on the adequate calcium intake continued to grow, including even the 18 months old dogs that had practically com-

pleted their growth at the beginning of the experiment. The animals on a low calcium diet without ammonium chloride gained weight more slowly after a time, although their growth in length seemingly continued. On the other hand, the dogs on the low calcium diet receiving ammonium chloride eventually developed anorexia and gained no weight. This was particularly noticeable in the youngest group where gain in weight ceased after the first 2 weeks on the low calcium diet with ammonium chloride. One animal developed deformities and fractures—a young puppy on low calcium diet, receiving ammonium chloride. The ammonium chloride effect was not as pronounced in the older animals. The animals receiving high calcium intakes and the ammonium chloride grew well.

### *Gross Pathologic Findings*

At autopsy the bones of the control dogs receiving the calcium supplement were normal in every respect.

They cut with usual resistance and the cortices were compact. The spongy trabeculae at the ends of the long tubular bones were entirely normal in appearance, and the internal architecture showed the usual arrangement of the trabeculae. The periosteum covering the long tubular bones stripped normally and the articular cartilages were smooth and glistening. The soft tissues showed no abnormalities in the gross, and the intestinal mucosae were intact throughout.

The animals of all age groups on a low calcium intake showed, on the other hand, readily discernible thinning of their bones, on comparison with their controls.

In Fig. 1 it is plainly visible that the cortex of the femur of the dog on the low calcium diet shows marked cortical thinning when compared with its adequate calcium control. Gross lamellation of the cortex with enlargement of the vessel canals is apparent. The general marrow cavity is enlarged. The spongy bone at the lower end of the femur extends over not quite as great a distance as does the spongy bone of the litter mate controls on the high calcium diet. The individual trabeculae are thinner, and this thinning of the trabeculae appears both in the extreme epiphyseal ends of the bone and in the metaphyses. The spongy trabeculae of the diaphysis have practically disappeared. Thus the effect of a low calcium intake is not limited to the spongy bone, but is reflected also in the cortex.

The macroscopic effects of the ammonium chloride treatment were observed in all the age groups, but were most striking in the younger

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The macroscopic effects of the ammonium chloride treatment were observed in all the age groups, but were most striking in the younger



animals. In all the age groups the gradations of change were found to be strikingly dependent upon the calcium intake. Those dogs receiving an adequate calcium intake and ammonium chloride showed less bone thinning than those receiving a low calcium diet and ammonium chloride.

Examination of Fig. 1 will show that in the 170 litter, the femoral cortex of the dog on a low calcium diet plus ammonium chloride is thinner than that of the dog on an adequate calcium diet plus ammonium chloride but not very much different from the low calcium control. The femoral cortices of both of these animals are definitely lamellated and the spongy bone of both the low calcium control and the low calcium plus ammonium chloride animals, while fairly abundant, consists of much more delicate trabeculae than observed in the metaphyses and epiphyses of the dogs receiving an adequate calcium intake plus ammonium chloride.

In the youngest age group the added effect of ammonium chloride brought out more striking changes than the low calcium diet alone.

In a dog put in the experiment at 3 months of age, a low calcium diet and ammonium chloride led to fractures and deformities. Less striking changes were observed in the litter mate given ammonium chloride and a high calcium diet. These differences are depicted in Fig. 2. The photograph shows a pronounced osteoporosis of the femur of the dog receiving an adequate calcium intake plus ammonium chloride; the cortex is extremely thin, about one-half to one-third that of its control. The spongy bone which is fine and delicate continues into the diaphysis but not as far as in the control; there are no deformities or fractures; the cortex shows definite gross lamellation. The litter mate on a low calcium intake with ammonium chloride suffered extensive fractures of the ribs, femora, and humeri. At autopsy great care had to be exercised in the removal of the bones to prevent further fractures because of the very marked friability. Healing of the fractures with callus proliferation and deformities was observed. The specimen shown in the figure is from the lower end of a macerated femur. The bone was so friable that some of it disintegrated in the process of maceration. Nevertheless the thin tissue-paper-like cortex is noticeable. The narrow deformed marrow cavity was closed in places by internal callus and the spongy trabeculae of the metaphyseal region were so soft and friable that careful handling had to be exercised to prevent the production of artefacts.

This also holds true for the 130 litter, shown in Fig. 3. The cortex of the femur of the dog on a low calcium intake plus ammonium chloride is very much thinner than that of the dog on an adequate calcium intake plus ammonium chloride and even somewhat thinner than the cortex of the low calcium control. The spongy trabeculae are thinner and the intertrabecular spaces wider in the animal on the

low calcium diet plus ammonium chloride than in the one which received an adequate calcium intake plus ammonium chloride. The spongy bone is even more delicate than in the low calcium control.

It was obvious in the gross as well as microscopically that rickets played no part in these experiments. Indeed our experience in this and in other series of tests has shown our meat diet, with the cod liver oil and tomato juice supplement, entirely adequate for growth and for prevention of deficiency diseases.

### *Microscopic Anatomy*

In the study of the ribs, comparative examinations were made of the various animals within the same group. We were careful to make comparisons on the basis of the severity of changes at equal distances from the costochondral junction.

Three of four ribs from each of the dogs were subjected to histologic examination. Generally the 6th, 7th, 8th, and 9th ribs were taken, and a large piece including the costochondral junction was examined. Fixation in Helly's fluid, decalcification in nitric acid, and staining with hematoxylin and eosin.

*18 Months Old Group.*—There were slight but readily distinguishable differences in the ribs of all the animals within this group, but these were not very marked. The control showed the most compact bone with the narrowest vessel canals and the fewest osteoclasts. The dogs on the adequate calcium intake plus ammonium chloride showed the least deviation from the normal. In them the vessel canals were already moderately enlarged but on the whole quite smooth, and osteoclasts were few in number. The dog on the low calcium diet and that on the low calcium diet plus ammonium chloride showed, in those ribs examined, the greatest change from the normal. Their ribs displayed the most marked enlargement of the vessel canals, and the greatest amount of subperiosteal and subendosteal resorption. It is, however, striking that the ribs in none of the dogs of this group presented marrow fibrosis.

*8 Months Old Group.*—In this group, the adequate calcium control had the most compact ribs. The histologic examination showed that maturity had not as yet been reached, and that there was active bone formation at the costochondral junctions. Naturally, the vessel canals of the cortex had not taken their final narrow size, though they were smooth walled and there was, except in the region of the costochondral junction, a minimum of subperiosteal and subendosteal resorption. The other two animals in this group showed a slight but definite deviation from the control, and the one on the low calcium diet with the ammonium chloride probably showed this change more than the one on the adequate calcium plus ammonium chloride. The differences between these two animals receiving

ammonium chloride was evident, although not very pronounced. These deviations from the normal consisted of increased size of the vessel canals, some increase of the connective tissue within these enlarged canals, and some increased subperiosteal and subendosteal resorption. In none of the animals of this group was the marrow fibrosed.

*6 Months Old Group.*—The histologic examination of the ribs showed a distinct separation of the animals into two classes—those whose bones were normal or approached the normal, and those whose bones were resorbed and considerably fibrosed. The calcium intake was the factor that governed the separation of the animals into these two groups.

The control dogs receiving an adequate calcium intake showed the usual minimal transformation changes, limited especially to the vicinity of the costochondral junctions. These are to be expected in growing transforming ribs in normal animals. The cortices of such ribs were more compact as they receded from the costochondral junctions. The vessel canals were of varying widths, but on the whole their walls were smooth and there were a minimal number of osteoclasts. The dogs receiving the adequate calcium diet plus ammonium chloride showed only the slightest histologic deviation from the control. In these the administration of ammonium chloride caused at the most a slight cortical thinning and some slight osteoclast increase in the enlarged Haversian canals. The marrow was not scarred in either these or the control.

The control receiving a low or inadequate calcium intake showed pronounced changes characterized by a pathologic exaggeration of the normal resorptive and transforming phenomena observed in the bones of growing dogs. The rib cortices throughout the sections were thinned to a very pronounced degree; the vessel canals were increased in diameter and filled with cellular connective tissue; the walls of the vessel canals showed many lacunae containing osteoclasts. There was in addition considerable subperiosteal and subendosteal resorption. The marrow was very extensively fibrosed and the intramedullary trabeculae were thinned, deformed, and showed evidences of active resorption. The proliferating cartilage zones were not widened and there was active resorption and fibrosis immediately beneath the growing zones.

The changes in the ribs of those dogs receiving this low or inadequate calcium intake plus ammonium chloride were entirely in the same direction as those observed in the low calcium control. The changes were of the same nature and distribution, and possibly of slightly greater extent.

*3 Months Old Group.*—In this group the adequate calcium control showed the expected transformation changes. Normal rib ossification was in progress, resulting in the production of a more compact cortex at distances away from the most active zones of transformation (costochondral junctions). The dog receiving adequate calcium and ammonium chloride showed slight but definite resorption in excess of that present in the normal animal. This was characterized by some subperiosteal and subendosteal resorption and some increased connective tissue

in the vessel canals with increased osteoclasts within these canals. Furthermore there was a very slight tendency to marrow fibrosis near the costochondral junctions.

The animal receiving the low calcium diet plus ammonium chloride showed very extensive resorptive and fibrotic changes. Every rib examined had fractures and infractions in various stages of healing. Some of the long bones showed fractures and deformities in various stages of healing. The effects of this régime caused scarring of the marrow, thinning of the cortices of the ribs, marked enlargement of the vessel canals with increased connective tissue in them. Numerous osteoclasts were observed.

### DISCUSSION

These experiments are in accord with the known fact that the administration of ammonium chloride induces porosis of bones. The age correlation is significantly demonstrated, as the effects of ammonium chloride diminish very materially as soon as the animal passes the young puppy stage. Smaller dosage per kilo sufficed to produce in young puppies very much more marked changes than could be produced by much larger doses in the adult.

The effect of ammonium chloride administration should not be considered, as it sometimes is, independently of the calcium intake or the calcium-phosphorus ratio. In this series of experiments, particularly in all animals 6 months and older, the level of calcium intake was most decisive in causing development or prevention of marked degrees of bone decalcification. In these dogs the differences between the low calcium and adequate calcium groups were frequently more obvious than the differences between dogs on an adequate calcium intake with and without ammonium chloride supplement. In the youngest puppies a definite effect of ammonium chloride in the adequate calcium group was evident, but even here the effect of the calcium withdrawal was considerably more drastic. The effects of administration of ammonium chloride with or without an adequate calcium intake led to gross fractures and deformities in only one of the youngest dogs. This animal received no supplementary calcium.

In none of these animals was there any evidence of rachitoid lesions which Bernhardt and Rabl (10) observed in young rats on a diet deficient in calcium and phosphorus, to which 2 per cent ammonium chloride was added as well as a small and, under the conditions of their experiment, possibly inadequate cod liver oil supplement.

The effects of ammonium chloride in causing an increased excretion of calcium are known. Furthermore the conclusion that the calcium comes from the skeleton is obvious. These facts have prompted certain clinicians to utilize ammonium chloride decalcification as a therapeutic vehicle towards the correction of bone deformities due to rickets (11). The ammonium chloride was generally given in doses of about 0.2 gm. per kilo body weight daily, but supplementary procedures, such as Bier's hyperemia daily for 15 to 20 hours, absolute rest in bed, and the application of constant traction and mechanical correction were used. It has been reported that by such non-surgical procedures some degree of correction of rachitic bow-legs is obtainable at a saving of time. It is more difficult to obtain these effects after the period of active rickets, and while osteoporosis may be obtained with ammonium chloride, it is more difficult to correct a deformity except by a long tedious usage of the auxiliary mechanical methods.

The histologic studies, furthermore, revealed a number of features that are helpful in understanding certain of the pathologic aspects of the general subject of osteoporosis. The feeding of an adequate calcium supplement led to a definite reduction in the rate of decalcification in all of our experiments. The older dogs receiving ammonium chloride and supplementary calcium showed a minimal number of osteoclasts and few Howship's lacunae. With an inadequate calcium intake and ammonium chloride, the ribs of such dogs showed increases in lacunae and osteoclasts and also in subperiosteal and subendosteal resorption. The same features held in the younger age groups whose animals receiving an adequate calcium intake tended to show less active resorption. In these animals also a low calcium intake led to the appearance of more osteoclasts and Howship's lacunae and the associated subperiosteal and subendosteal resorption. In the younger animals calcium insufficiency was found to be distinctly responsible for the rapidity with which the histologic pictures of resorption appeared, and for their severity.

When the decalcification was rapid, such degrees of marrow fibrosis and such extensive bone resorption resulted that the histologic picture could be designated as generalized osteitis fibrosa. The youngest animals in our experiments receiving an inadequate calcium intake plus ammonium chloride developed such lesions, while the older ones on

the same régime only developed what could be called simple osteoporosis.

This difference in effect in the various age groups brings up the question of what is to be comprehended by the term *ostitis fibrosa*, and also the still more important question of relationship to each other of the *ostitides fibrosae* produced by various procedures. We have previously reported on the production of *ostitis fibrosa* in guinea pigs and dogs through the administration of parathormone. These lesions are in a broad sense of the same histologic appearance as those that are produced in young actively growing dogs on a low calcium but otherwise adequate diet, although other important differences were noted. The administration of ammonium chloride to a puppy receiving an inadequate calcium diet will lead, when the diet is so protected that none of the vitamin deficiencies will result, to generalized *ostitis fibrosa*, histologically quite indistinguishable from that which results from an inadequate calcium intake alone. Furthermore, the amounts of calcium adequate for slow growth will, if these puppies are very young and actively growing, produce a histologic picture of *ostitis fibrosa*, different only in quantitative aspects from that which appears in a rapidly growing puppy on an inadequate calcium intake.

While the *ostitides fibrosae* produced by the above mentioned procedures lead to basically the same histologic pictures, the gross appearances of the dogs subjected to these varying régimes will be different. Dogs on low calcium intake, receiving parathormone or relatively large doses of ammonium chloride will show severe *ostitis fibrosa* associated with stunting and deformities. The stunting is due primarily to loss of appetite. It is significant therefore that a severe *ostitis fibrosa* will develop under the influence of ammonium chloride or parathormone in such animals whose growth has practically stopped. The *ostitis fibrosa* is here clearly attributable to a drastic decalcification unaided by the demands of rapid growth. In simple calcium deficiency a similar picture of *ostitis fibrosa* will be produced in an animal in which the calcium deficiency is aggravated not by parathormone nor by ammonium chloride, but by the physiological demands of bone growth.

We must conceive of generalized *ostitis fibrosa* as a rather inclusive category, embracing not only the clinical generalized form (von Reck-

linghausen's disease), which has a specific parathyroid etiology, and the experimentally produced *ostitis fibrosa* of similar origin, but also the experimentally produced conditions which are caused by other means leading to very rapid calcium depletion with resulting marked marrow connective tissue proliferation. Thus, either clinically or experimentally, if bone decalcification is very rapid—and this would hold in the young or in the adult—generalized *ostitis fibrosa* occurs. From this point on there may be modification of both the gross and histologic features of the *ostitis fibrosa*, due to the special underlying factor inducing the decalcification. Thus it is conceivable that hyperparathyroidism may in the course of the development of *ostitis fibrosa* favor incidentally very extensive hemorrhage into the marrow, resulting in brown blood cysts and the formation of giant cell tumors. It has been recently shown (12) that generalized *ostitis fibrosa* may exist without the other classical features of von Recklinghausen's disease (cysts and giant cell tumors) apparently on the basis of a hyperparathyroidism, as at autopsy parathyroid adenomas were found.

On the other hand, a slowly developing decalcification from whatever cause will lead, both in the young and adult, to a less severe lesion which is called osteoporosis, because of the absence of striking fibrosis. The occurrence of osteoporosis in adults under a number of circumstances is well known and one of the most common causes for it is Graves' disease. In this disease there is negative mineral balance, but the patients do not suffer from the effects of diminished calcium intake. The disease is chronic and the decalcification is slow but progressive.

In long tubular bones the same phenomena are observed. Relatively rapid decalcification has been found to occur in the metaphysis—the region of rapid bone growth and metabolism,—and slow decalcification in the diaphysis and epiphysis. Corresponding to these changes are the histologic pictures of *ostitis fibrosa* and osteoporosis observed in the respective portions of such long bones (6).

The modifying effects of several factors are also illustrated in experimental rickets. It is well known that rickets cannot be produced experimentally in rats by withholding vitamin D unless there is a simultaneous mineral imbalance (disturbance of calcium-phosphorus ratio). In very young rapidly growing rats with curative

vitamin D supplement, but on a low calcium diet, an ostitis fibrosa will develop. On the same régime with 1000 to 2000 rat units of vitamin D, the marrow fibrosis will be essentially prevented and simple osteoporosis will ensue. The same diet with the exclusion of vitamin D will produce rickets.

While these experiments have yielded comparisons of low calcium and high calcium régimes, it is recognized that similar results might have been obtained with a different type of calcium-phosphorus imbalance—namely on low phosphorus diets.

#### CONCLUSIONS

1. In all age groups the effects of ammonium chloride administration were found to be strikingly dependent upon the calcium intake.

2. Dogs receiving an adequate calcium diet and ammonium chloride showed less decalcification than those receiving a low calcium diet with or without ammonium chloride.

3. In the younger groups the added effect of ammonium chloride to calcium-low diet brought out more striking changes than a low calcium diet alone.

4. When the decalcification was less severe—in the oldest dogs on the low calcium diet with or without ammonium chloride, and in the younger dogs on an adequate calcium intake with ammonium chloride—generalized thinning of the bones without marrow fibrosis resulted (osteoporosis).

5. When the decalcification was rapid and severe—in the youngest dogs on low calcium diet, particularly with ammonium chloride—generalized decalcification and secondary marrow fibrosis resulted (ostitis fibrosa).

6. Generalized ostitis fibrosa is a rather inclusive term and may be applied to the histologic picture which results when clinical or experimental decalcification is rapid, and therefore leads to extensive marrow fibrosis.

7. The special underlying causes of the decalcification may incidentally contribute features to modify the generalized osteoporosis or ostitis fibrosa as in rickets and in von Recklinghausen's disease.

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## EXPLANATION OF PLATES

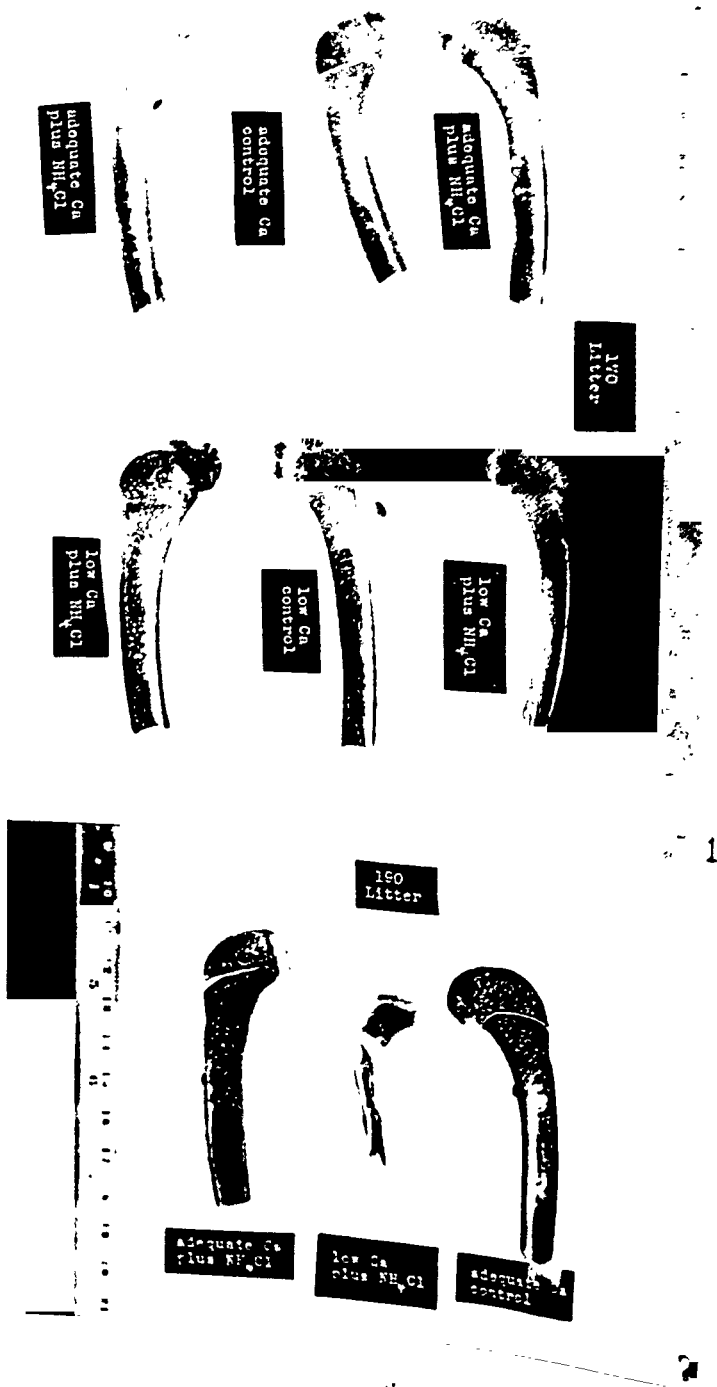
## PLATE 41

FIG. 1. Photograph of the distal portions of the femora of the 6 months old dogs. Macerated specimens.

FIG. 2. Photographs of the distal portions of the femora of the 3 months old dogs. Macerated specimens.

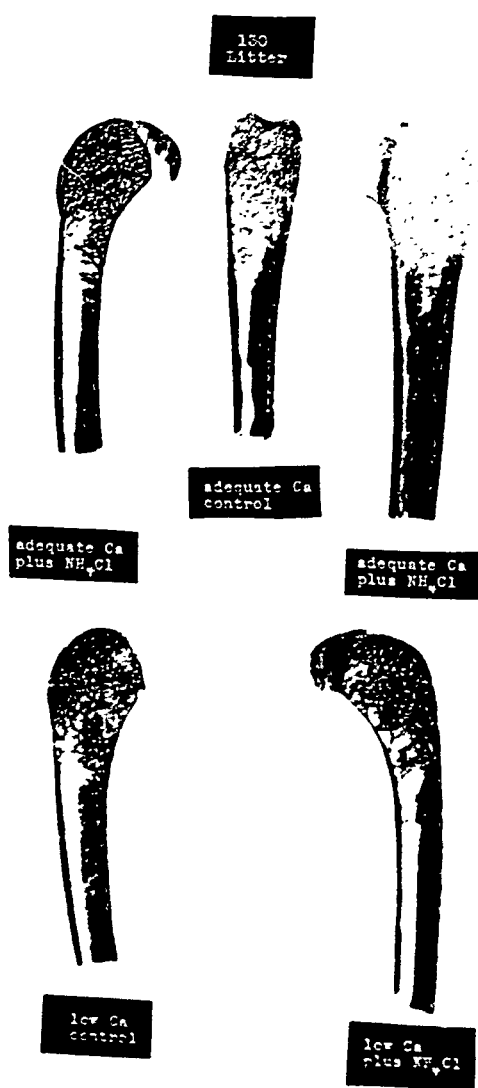
## PLATE 42

FIG. 3. Photographs of the distal portions of the femora of the 18 months old dogs. Macerated specimens.

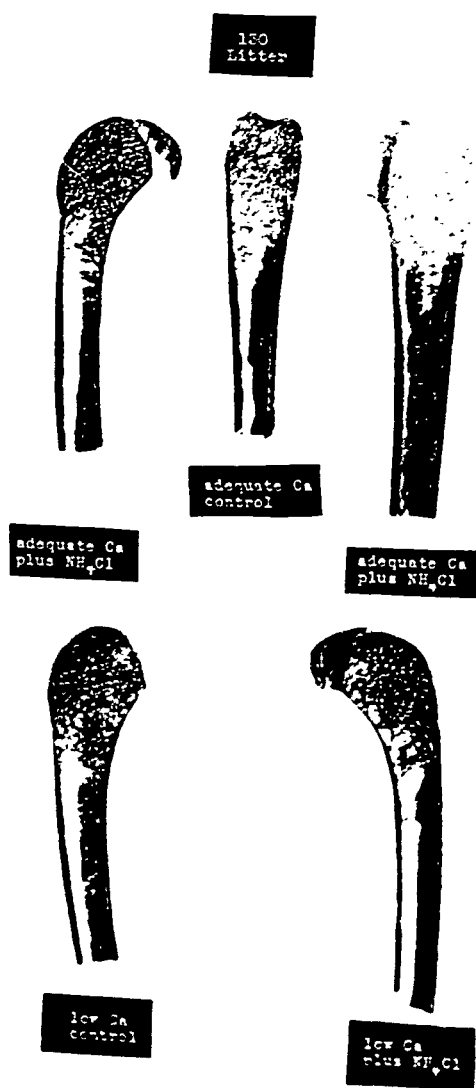


(Jaffe et al.: Ammonium chloride decalcification)









(Note:  $\text{NH}_4\text{Cl}$  causes de-calcification)



# THE MATERNAL TRANSMISSION OF VACCINIAL IMMUNITY IN SWINE

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PLATE 43

(Received for publication, June 30, 1932)

The route by which antibodies are transmitted from an immune female animal to her young has been determined for most of the domesticated and laboratory animals. There are comparatively few observations, however, on the maternal transmission of protective substances, aside from the antitoxins. In the case of antibodies the path of conveyance is either a direct one by way of the placenta or an indirect one by way of the mother's colostrum or milk. As pointed out by Kuttner and Ratner (1) the direct passage of antibodies is influenced by the histologic structure of the placenta. Whether or not the same relations are applicable to the transmission of protective substances can be decided only by actual test. In the cow, with a transitional type of placenta, it was shown by Smith and Little (2), in their work on calf scours, that protective substances were conveyed by the colostrum. It was already known that antibodies were transferred from cow to calf by the indirect route. The present work with swine was undertaken to determine the route of transmission in an animal with a different placental structure, specifically a true adeciduous placenta.

Considerable attention has been paid to the transmission of protection in swine by reason of its bearing on vaccination procedures in hog cholera. It was recognized from field experience and demonstrated in controlled experiments by McArthur (3) and by Pickens, Welsh, and Poelma (4) that the young of sows immune to hog cholera were temporarily resistant. This resistance was generally attributed to a transfer through the colostrum or milk but the possibility of placental transmission was not definitely excluded. The route of antibody transmission was worked out by Connaway (5) who in a study of swine abortion found that the colostrum was the chief vehicle of conveyance.



TenBroeck and Ring (6) had recently shown that it was possible to take newborn pigs from their dams, before suckling, and raise them on a mixture of commercial cow's milk powder and normal swine serum. The controls essential for ruling out a placental transmission, which were lacking in the earlier work on hog cholera, could be supplied by this procedure.

Vaccinia virus was selected as the immunizing agent for the demonstration of maternal transmission. Swine were found to be naturally susceptible to vaccinia, responding to the presence of virus in the skin with the usual vesicular reaction. The first manifestation, after vaccination, was a slight elevation of the skin with a little congestion, appearing generally on the 2nd day. Definite papules, discrete or confluent, developed on the 3rd or 4th day. These increased in size and usually became vesicular a day later. At this time the lesion appeared as a white-tipped nodule measuring up to 10 mm. at the surface and generally surrounded by a red zone of congestion. In some cases the vesicles persisted for an additional day, rarely longer, but usually scab formation was visible on the 6th or 7th day. The swelling rapidly subsided, the congestion faded, and after a variable period the scabs fell off. The reaction period, it may be noted, is considerably shorter than in man and there is no permanent scar formation.

### *Methods*

Young sows in their first or second pregnancy were vaccinated with vaccinia virus immediately before or shortly after breeding.<sup>1</sup> The vaccine was introduced into the superficial layers of the skin over the inner surface of the flank, an area which is relatively hairless, clean, and protected. With adult animals the skin was washed with alcohol and dried with ether. This procedure was generally omitted in the vaccination of young animals with no unfavorable results. Two parallel scratches, approximately 2 inches long and 1/2 inch apart were made in the skin with a pointed hypodermic needle. Three drops of vaccine fluid were placed along each line with a capillary pipette and rubbed into the abraded area.

The gestation period in swine, approximately 115 days, allows ample time for the development of a solid immunity. The vaccinated sows were kept in confinement towards the close of the estimated gestation period and closely watched. At parturition, their young were taken before they had suckled and divided into two groups. One group was subsequently returned to the sow and allowed to nurse. The pigs of the other group were fed by hand a mixture of dried cow's

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<sup>1</sup> The vaccine employed in this work was obtained from the Laboratories of the New York City Department of Health through the courtesy of Dr. W. H. Park.

milk and normal swine serum. On the 7th day, in most instances, the young pigs were vaccinated and kept under observation for 10 days or more.

The experimental findings with the young of three vaccinated sows are presented in detail in the following case reports and summarized in Table I. The cutaneous reaction in suckling and hand-fed pigs, following vaccination, is shown in Figs. 1 and 2.

TABLE I

*The Reaction to Vaccinia Virus in the Suckling and Hand-Fed Young of Immune Sows*

Sow No.	Date of vaccination	First litter			Second litter		Third litter		
		Date of birth	No. of suckling pigs vaccinated and result	No. of hand-fed pigs vaccinated and result	Date of birth	No. of suckling pigs vaccinated and result	Date of birth	No. of suckling pigs vaccinated and result	No. of hand-fed pigs vaccinated and result
	1930	1931			1931		1932		
1	Dec. 18	Apr. 6	3 - *	4 +	Oct. 1	3 -	Mar. 20	3 -	
2	Nov. 6	May 1	3 -	1 +	Oct. 18	4 -	Apr. 14	3 -	3 +
	1931				1932				
3	July 7	Oct. 29	1 - 1 ±	2 +	Mar. 25	3 -			

\* - = no reaction; ± = papular reaction; + = vesicular reaction.

### *Case Reports of the Suckling and Hand-Fed Pigs from Vaccinated Sows*

Sow 1 was born Mar. 28, 1930; bred for the first time Dec. 16; and vaccinated Dec. 18 with a typical vesicular reaction. A litter of seven pigs was born Apr. 6, 1931. Three young were placed with the dam and allowed to suckle. Four were fed by hand. The pigs of both groups were vaccinated Apr. 13. The three suckling pigs failed to react during a period of 14 days. The four hand-fed pigs showed vesicle formation on the 4th day. One pig died at this time. The three remaining pigs showed beginning scab formation on the 8th day. A suckling pig, of approximately the same age, from a non-immune sow was vaccinated at the same time and reacted typically. Scab formation began on the 6th day.

Sow 2 was born Apr. 8, 1930; vaccinated Nov. 6; and bred Feb. 10, 1931, for the first time. A litter of nine pigs was born May 1. Three young were placed with their dam to nurse and six were fed by hand. All of the young were vaccinated May 5. The three suckling pigs showed no reaction through a period of 2 weeks.

Five of the hand-fed pigs died during the first 48 hours after vaccination. The remaining hand-fed pig showed vesicle formation on the 6th day and scabbing on the 9th. A suckling control pig farrowed by a non-immune sow was likewise vaccinated and reacted with the formation of vesicles which began to scab on the 7th day.

Sow 3 was born Mar. 28, 1930, a litter mate of No. 1; vaccinated July 7, 1931, with a typical reaction; and bred for the second time July 10. Four pigs were born Oct. 29. Two of them nursed their mother and two were fed by hand. They were vaccinated Nov. 5. One suckling pig showed no reaction during a 10 day interval. The other developed several papules on the 3rd day after vaccination. These began to scab on the 5th day without vesicle formation. The two hand-fed pigs reacted with typical vesicles which appeared on the 4th day and began to scab on the 7th.

The three immune sows were rebred and the young of their second pregnancies, after vaccination, were tested for protection to vaccinia virus. The new-born pigs which numbered six, eight, and four, respectively, were all placed with their dams and allowed to nurse. On the 7th day after birth, three young from the first sow, four from the second, and three from the third were vaccinated and kept under observation for 10 days. In no case was any local reaction visible during this time. Two control pigs, the suckling young of non-immune sows, reacted with the formation of typical vesicles.

The first two sows were rebred for the third time. They farrowed during the early spring of 1932, approximately 15 months after they were vaccinated. Sow 1 had a litter of six pigs, all of which were allowed to suckle. Three of the nursing young were vaccinated without "take" on the 7th day. Eleven pigs were farrowed by Sow 2. Three of this litter were allowed to suckle and eight were fed by hand. Five of the latter group died before the 7th day of life. The other three were vaccinated at that time and showed typical vesicular reactions.

#### DISCUSSION

The combined vaccinated young of successive litters farrowed by the three immune sows numbered 34, of which 24 were suckled pigs and 10 were fed by hand. Twenty-three of the first group showed no reaction to vaccinia virus introduced into the skin. They were completely protected against the concentration of virus used for vaccination. The acquired resistance, as will be shown at a later time, was of relatively short duration. One suckling pig displayed an incomplete reaction, which progressed only to the formation of papules, indicative of a partial protection. The hand-fed pigs all responded to the presence of virus with the formation of typical vesicles. The only observed difference in the reactions of hand-fed pigs and the suckling young of non-

immune dams was the somewhat delayed appearance of scabs in the case of the former. One pig in the group of suckling young whose dams were unprotected was farrowed by Sow 3 prior to vaccination. The susceptibility of this pig to vaccinia contrasted sharply with the resistance of the suckling young from the same sow following vaccination.

In spite of a considerable loss of protective substance at each parturition, two of the vaccinated sows continued to transmit protection to the suckling young of three consecutive pregnancies. The suckled pigs of the two litters showed no significant difference in the degree of resistance acquired by the ingestion of their dams' colostrum. The time interval between the vaccination of the sow and the test vaccination of the third pregnancy young was approximately 15 months. The sow, meanwhile, had received no additional virus from an outside source.

These observations indicate that protection against vaccinia in swine, initiated by the cutaneous introduction of virus, may be transmitted from sow to young. They show, moreover, that the porcine placenta is impermeable to any appreciable amount of protective substance and that the function of immunity transfer is taken over by the colostrum. These conclusions coincide with those pertaining to the maternal transmission of antibodies in swine and suggest that similar controlling factors are involved.

#### SUMMARY

The introduction of vaccinia virus into the skin of swine calls forth a typical vesicular reaction which may be followed by a solid immunity. This acquired state of resistance was utilized in determining the route of immunity transmission from sow to young. The suckling young of immune sows, vaccinated on the 7th day or earlier, showed no reaction to the virus. Their hand-fed litter mates, however, were susceptible and reacted with the formation of vesicles. These observations indicate that the porcine placenta is largely impermeable to protective substances and establish the fact that colostrum functions as the vehicle for their transmission as it does for antibodies.

The writer is indebted to Mr. E. R. Ring for his meticulous supervision of the feeding and handling of the young pigs.

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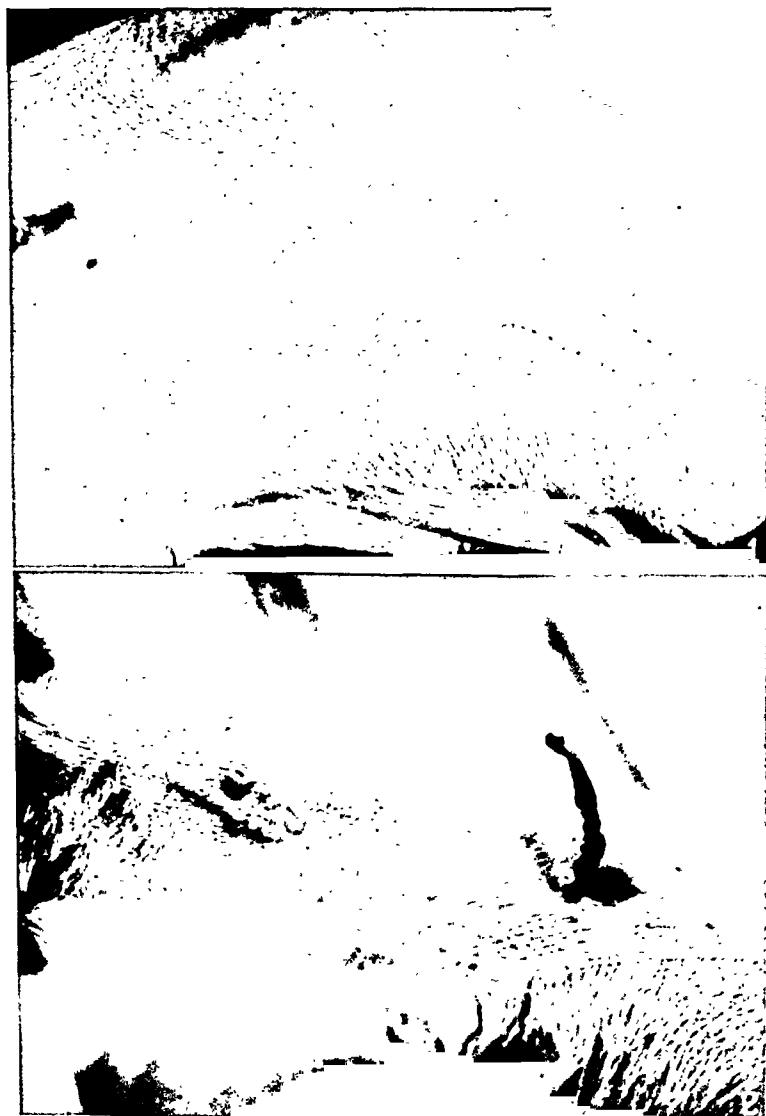
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## EXPLANATION OF PLATE 43

The skin reactions, on the 4th day after vaccination, in young pigs from the third litter of Sow 2.

FIG. 1. Suckling young with only the scab covering the original scratches visible.

FIG. 2. Hand-fed young with discrete and confluent vesicles visible along both scratches.



(Nelson: Transference of immunity in syngene)

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FIG. 2. Hand-fed young with discrete and confluent vesicles visible along both scratches.



(Nelson: Transmission of immunity in swine)





# STUDIES ON THE BLOOD CYTOLOGY OF THE RABBIT

## IX. BLOOD PLATELET COUNTS ON HEALTHY MALE RABBITS

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(Received for publication, July 14, 1932)

During a study on the hematological constitution of the rabbit under various conditions and circumstances (1-8), observations were made, as opportunity permitted, on the platelet count of the peripheral blood of healthy rabbits. The animals, as far as could be determined by continued inspection and body weight values, were free from disease conditions, such as snuffles, which may occur in the laboratory stock. Similar observations carried out on other rabbits which could not be considered healthy according to these criteria have been excluded from analysis. Within the prescribed limits, therefore, it may be assumed that the results obtained and herewith presented represent a fair sample of the platelet values of healthy laboratory rabbits.

### *Material and Methods*

This study is based upon 991 platelet determinations made on 148 male rabbits during the 2½ year period between Nov. 8, 1929, and May 15, 1932. All animals were in good physical condition and apparently free from any disease during the entire period of observation which continued for 3 weeks after the last count. The criteria of health included the features of nutrition and weight, as well as vigor, alertness, appetite, and condition of the fur. The occurrence of such conditions as a purulent nasal discharge, ear canker, subcutaneous abscess, or diarrhea was considered sufficient to eliminate the counts on such an animal from the present analysis. The results include all counts made on each animal fulfilling the above requirements.

Rabbits from two sources were employed: those purchased from dealers and those bred in the laboratory. The animals purchased from dealers were representative of the types commonly employed, that is, greys, browns, and Flemish crosses with an occasional black or albino. The rabbits bred in the laboratory consisted of pure and of hybrid types (Table I). The animals were 4 to 12 months of age; 6 were 18 months old.

Each animal was kept in an individual cage in a well ventilated, sunny room.

The diet from Nov., 1929, to Sept., 1930, consisted of hay, oats, and cabbage. In Sept., 1930, the cabbage was replaced by artificial food pellets and a free supply of water.

TABLE I

*991 Blood Platelet Counts upon 148 Healthy Male Rabbits Distributed According to Animal Breeds*

Breed (pure)	No. of rabbits	No. of counts	Breed (hybrids)	No. of rabbits	No. of counts
Sable	2	8	Albino-Himalayan	17	75
Silver	2	10	Dutch-Himalayan-	5	17
Chinchilla	5	25	Albino		
Polish	6	30	Lilac-English	1	2
Havana	13	59	(Purchased from	29	474
English	18	81	dealers—composition unknown.)		
Dutch	10	44	Brown, grey, etc.		
Beveren	10	48			
Gouda	5	23			
Himalayan	19	68			
Belgian	6	27			
Total.....	96	423	Total.....	52	568

Fifteen groups of rabbits were examined as shown in Table II. Elimination of diseased animals is responsible for the smaller number of animals comprising many of the groups. The actual number of animals eliminated is as follows: Group I, 5; Group II, 6; Group III, 4; Group IV, 8; Group V, 3; Group VII, 3; Group VIII, 6; Group XV, 10 rabbits respectively.

In general, 4 to 5 platelet counts were made on an animal, but the intervals between counts were variable. From the standpoint of the time interval, the animal groups fall into three categories. The first is represented by Group V (Table II) in which regular weekly blood examinations were made over an initial period of 9 months and biweekly over a subsequent period of 8 months. The second is represented by Groups I, II, III, IV, VI, XI, XII, XIII, XIV, and XV in which 2 to 5 counts were spread over a period of 1 to 2 weeks. The third category is represented by Groups VII, VIII, IX, and X, on each animal of which 4 successive platelet counts were made on the same day from the same ear vein incision.

In Table III is shown the distribution of the animals and counts by months. During the 2½ year period between Nov., 1929, and May, 1932, platelet counts were made in the following 21 months: Nov. and Dec., 1929; Jan., Oct., Nov., Dec., 1930; Jan., Feb., Mar., Apr., May, June, Sept., Oct., Nov., Dec., 1931; and in Jan., Feb., Mar., Apr., May, 1932. No counts were made during the months of July and August, and only once in June and September. During these 21 months

TABLE II

*Data on Animal Material Used for Platelet Counts*

Source of animals	Group No.	No. of animals	Date of first count	Date of last count	No. of counts
Purchased from dealers	I	7	Nov. 8, 1929	Nov. 19, 1929	28
	II	6	Dec. 31, 1929	Jan. 14, 1930	30
	III	2	Jan. 15, 1930	Jan. 17, 1930	4
	IV	7	Oct. 30, 1930	Nov. 13, 1930	21
	V	7	Oct. 30, 1930	May 15, 1932	391*
Bred in the laboratory	VI	8	Dec. 31, 1929	Jan. 14, 1930	40
	VII	11	Mar. 24-25, 1931	Mar. 24-25, 1931	36†
	VIII	5	May 19-20, 1931	May 19-20, 1931	20
	IX	10	June 10-12, 1931	June 10-12, 1931	40
	X	10	Sept. 17-18, 1931	Sept. 17-18, 1931	39‡
	XI	6	Apr. 21, 1931	Apr. 28, 1931	13
	XII	10	Nov. 5, 1931	Dec. 3, 1931	50
	XIII	5	Mar. 24, 1932	Mar. 29, 1932	32
	XIV	23	Mar. 22, 1932	Mar. 30, 1932	92
	XV	31	Apr. 12, 1932	Apr. 26, 1932	155
From dealers.....	5	29	Nov. 8, 1929	May 15, 1932	474
Laboratory bred.....	10	119	Dec. 31, 1929	Apr. 26, 1932	517
Total.....	15	148	Nov. 8, 1929	Apr. 26, 1932	991

\* One animal died through accident.

† 8 counts lost.

‡ 1 count lost.

TABLE III

*991 Blood Platelet Counts upon 148 Healthy Male Rabbits Distributed According to Months (1929-32)*

Month	No. of rabbits	No. of counts	Month	No. of rabbits	No. of counts
Sept.	10	39	Mar.	45	207
Oct.	14	38	Apr.	43	229
Nov.	31	127	May	15	57
Dec.	31	83	June	16	52
Jan.	23	119	July	0	0
Feb.	7	40	Aug.	0	0
Total.....				148	991

blood for platelet counting was taken on 90 different days, usually Tuesdays, Thursdays, or Fridays. Approximately 11 platelet counts were made, therefore, on each day on which animals were bled.

The observations were usually made between 9 and 12 a.m., but a large number were also made between 2 and 4 p.m., and the examinations were always carried out on animals which had received no food since the previous day. The platelet values were determined according to the method described by Casey and Helmer

TABLE IV  
*Blood Platelet Counts on Healthy Rabbits*

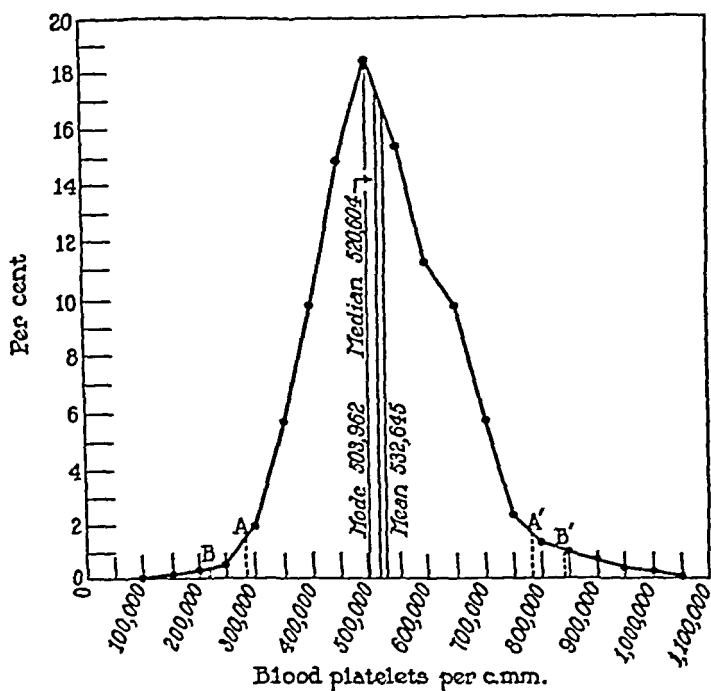
Mean of class	Frequency of counts	Frequency <i>per cent</i>	Summary of observed and estimated values	
150,000	1	0.1	Mean	= 532,645 per c.mm.
200,000	3	0.3	Median	= 520,604 per c.mm.
250,000	5	0.5	Mode	= 503,962 per c.mm.
300,000	20	2.0	Maximum	= 1,120,000 per c.mm.
350,000	56	5.7	Minimum	= 170,000 per c.mm.
400,000	97	9.8	Standard deviation	= 124,960 per c.mm.
450,000	148	14.9	Standard error of	
500,000	182	18.4	the mean	= 3,970 per c.mm.
550,000	153	15.4	Standard error of	
600,000	111	11.2	the standard de-	
650,000	97	9.8	viation	= 2,808 per c.mm.
700,000	56	5.7	$\gamma_1$	= $+0.558 \pm 0.078$
750,000	24	2.4	$\gamma_2$	= $+0.872 \pm 0.156$
800,000	14	1.4	Coefficient of vari-	
850,000	10	1.0	ation	= 23.46 per cent
900,000	7	0.7		
950,000	4	0.4		
1,000,000	2	0.2		
1,050,000	0	0.0		
1,100,000	1	0.1		

(9) and by Casey (10) in which Ringer's solution, containing a small amount of heparin, is used as the diluent. The blood is taken up in an ordinary red blood cell pipette and a red blood cell count made in the usual manner with a Neubauer chamber. After the red blood cells have been counted, the blood platelets are counted in the same preparation with the low power dry lens; the platelets appear as black refractile bodies about one-tenth to one-half the size of a red blood cell. The technical error<sup>1</sup> in this procedure has been found to be 8 per cent which is

<sup>1</sup> The coefficient of variation due solely to technique is called the technical error in this instance.

smaller than those found in any of the other methods tested and compares with a 5 per cent error for red cell and an 11 per cent error for white cell determinations.

The usual statistical methods were employed in analyzing the results obtained (11-13). A difference was considered to be significant when it was more than  $2\frac{1}{2}$  times its standard error; that is, when the probability of its occurrence by chance was less than 1 in 100. The mode was calculated by an application of King's formula.



TEXT-FIG. 1

## RESULTS

The results of the statistical analysis of 991 blood platelet counts on 148 healthy male rabbits include estimated values for the mean, the mode, the median, the maximum, the minimum, the standard deviation, the standard error of the mean and standard deviation, and the coefficient of variation (Table IV, Text-fig. 1). The value  $\gamma_1$  indicates that the distribution curve resulting from the particular determinations selected is significantly skewed to the right. The value  $\gamma_2$  indicates that this distribution does not follow the normal curve of

error, being heaped up too high on the top and cut too thin at the shoulders.

The lines marked A and A' in Text-fig. 1 are separated from the mean value of 533,000 by an interval equal to twice the standard deviation. The lines marked B and B' are separated from the mean by an interval equal to  $2\frac{1}{2}$  times the standard deviation. This signifies that counts less than the value represented by A or greater than the value represented by A' should not be expected to occur among healthy animals (if the animals selected constitute a fair sample and the real distribution is normal) more often than once in 22 counts. In like manner, values less than B or greater than B' should not be expected to occur more often than once in 100 counts. On this basis one might expect a platelet count greater than 1,000,000 per c.mm. or less than 100,000 to occur but once in 1,000 counts among healthy male rabbits.

The results of the present study indicate that a platelet count of more than 280,000 and less than 780,000 per c.mm. should be considered as normal while values of less than 220,000 or more than 820,000 per c.mm. should be designated as abnormal, regardless of the breed, time of the year, or other factors.

#### DISCUSSION

If platelet counts were made on representative types from all the standard breeds of rabbits and their hybrids, such counts being done at intervals of the day, week, month, year, and era; under various conditions of diet and housing; in different localities in the world, with various technical methods, a relatively accurate analysis of the blood platelet count in healthy adult male rabbits might be attempted. Since such extensive information is difficult to obtain, any one group of workers must strike some sort of compromise and then strive to evaluate the dependability of the results obtained.

Under Material and Methods some pains were taken to describe fully just what sort of compromise was entered into in collecting the present series of counts. It is now necessary to attempt an evaluation of these points of compromise.

*Time.*—The time of the day, week, month, and year in which the platelet counts were made might affect the results obtained. As to time of the day, the blood was taken between 9 and 12 a.m. and be-

tween 2 and 4 p.m. In an investigation of the material two groups of rabbits with 13 and 11 animals respectively were counted on 4 days distributed over a period of 2 weeks. It so happened that Group I was counted between 9 and 12 a.m. on the 1st and 3rd days and between 2 and 4 p.m. on the 2nd and 4th days; Group II, on the other hand, was bled between 2 and 4 p.m. on the 1st and 3rd days and between 9 and 12 a.m. on the 2nd and 4th days. When all counts (48) made in the morning were averaged and compared with all counts made in the afternoon (48), a difference of  $3,917 \pm 12,768$  existed between the respective means. Since the difference between morning and afternoon counts was less than twice its standard error, it would not seem likely that such variations as might occur during the remaining 12 hours of the day would materially alter the population figures for platelet counts obtained.

As to day of the week, counts were made on 90 different days during this  $2\frac{1}{2}$  year period particularly on Tuesday, Wednesday, Thursday, and Friday, but also to some extent on Monday and Saturday. The day of the week, therefore, would not be expected to offer particular bias in the results obtained.

As to the month of the year, means were calculated of all counts made in each of the 10 months followed. These ten monthly means were added and a mean for the entire 10 months of  $524,759 \pm 12,348$  obtained. This differs from the general means of 532,645 by  $7,876 \pm 13,524$ . Since the difference was less than its standard error, even with the addition of the estimated values for July and August, the general mean if calculated by months would not be significantly different from the mean obtained upon the 991 counts.

*Breed.*—Of the 30 or more recognized standard breeds of rabbits about 15 are of medium or small body build and most of these are represented in our series. Counts were made also upon the heavier breeds such as New Zealand, American Blue, Flemish Giant, but these animals adapted poorly to cage life and were not in good physical condition. Their counts were eliminated from this series.

The means of all the counts made on each breed were calculated and from these 15 breed means a grand mean of 559,000 was obtained. This differed from the general mean by  $26,995 \pm 13,524$ . Since it is not quite twice the standard error of the difference, the disproportion-



ate number of counts on certain breeds did not seem to significantly affect the results obtained. Also the breeds of animals showing highest platelet counts were nearly all counted in the months of the year when the blood platelets are highest; therefore, the blood platelet level for breeds is possibly higher than it might be if seasons of the year were held constant.

The possible difference between pure breeds and hybrids was also considered. Analysis of mean platelet counts upon 96 pure breed rabbits was  $545,687 \pm 8,564$  and upon the 23 laboratory bred hybrids  $549,551 \pm 17,876$ . The difference of  $3,864 \pm 19,821$  did not indicate a difference in pure breeds and hybrids which would materially alter the values obtained.

*Source of Animals.*—It was stated that 29 animals were purchased from dealers whereas the remaining animals were bred in the laboratory. The mean platelet counts in the 29 rabbits obtained from dealers was  $523,517 \pm 17,243$ . This differed from the mean for the 119 laboratory bred rabbits ( $546,434 \pm 7,622$ ) by  $22,917 \pm 18,830$  and was not significant.

*Means for Individual Animals.*—Since a large number of counts were made upon each of a single group of 6 animals, the question arose as to how far the mean and standard deviation might be affected by this type of bias. To this end the mean platelet count was obtained for each of the 148 rabbits in this series. A general mean was then obtained for these 148 means and found to be  $543,245 \pm 6,915$ . This differed by  $10,000 \pm 7,973$  from the mean of the 991 counts in the same animals and was not significantly different. Again the standard deviation of the 517 counts made on laboratory bred rabbits did not differ from the standard deviation obtained for the entire 991 counts. It was concluded, therefore, that the large number of counts upon the one group did not materially affect the mean and standard deviation obtained.

The rabbits were selected on the basis of physical fitness and apparent freedom from disease. Our ability to select appropriate animals, therefore, entered into the results obtained. It is possible and also probable that some animals with internal disease conditions may have been included. The question arises as to whether the skew to the right in the frequency curve is due to the inclusion of such individuals.

The only available information on this point is indirect. The mean of all platelet counts on the 48 rabbits eliminated from this series because of visible disease or malnutrition was 655,188 per c.mm., that is, a value which exceeds by 122,543 the mean of the apparently normal animals. This difference which is more than 6 times the standard error of the difference is, therefore, highly significant. When the platelet counts on these 48 externally diseased rabbits were included in the frequency curve, together with those of the normal rabbits, the mode was not affected (503,962) but the mean was significantly higher (560,471) and the skew to the right in the curve was marked. The removal of the counts on the diseased animals from the frequency curve greatly reduced the skew, and it is possible, therefore, that the skew would have been further reduced if the animal material could have been more rigorously selected.

*Diet.*—It was mentioned in the section on Material and Methods that the diet was altered in September, 1930. The mean of all platelet counts (102) on normal animals (23) made prior to the change was  $526,400 \pm 12,727$  and of (205) counts on (24) rabbits made after the change in the same 3 months of November, December, and January,  $521,465 \pm 8,474$  per c.mm. respectively. The difference between the two means,  $4,935 \pm 15,290$ , is less than twice the standard error of the difference, so that the change in the diet cannot be said to have significantly affected the mean values of the platelet counts.

It should be pointed out that although no significant changes occurred in platelet values of the present series which could be ascribed to differences in source of animals, diet, or method of counting, it is possible or perhaps probable that such related changes could occur. The experiments were not arranged to bring out such differences, but, on the contrary, to minimize them if they were present. As far as can be ascertained at present, all that one can say is that in this series no individual factor appeared to exert a significant bias in the results.

Other features of the technique employed which might affect the results were the fact that the counts were made on fasting animals and that the procedure of platelet enumeration was different from that commonly used. Conditions of housing and climate and the years in which the study was made are other factors which theoretically might

be considered. The results of other workers using different methods of platelet counting as well as different experimental conditions are, however, comparable to ours, and it would appear, therefore, that in the usual circumstances of the laboratory and with the usual rabbit stock, no striking differences in mean platelet values are to be expected. The values compiled by Scarborough (14) in 1926 from the results of ten authors, most of whom used different methods and made counts on fewer than 20 animals, averaged 540,000 platelets per c.mm. with limits of from 200,000 to 1,000,000. Dudgeon and Goadby's (15) recent platelet values for 3 normal rabbits were 503,400, 489,000, and 1,009,200 per c.mm.; Ecker and Rees (16) gave platelet values for 8 normal rabbits which averaged 559,000 and ranged from 368,000 to 853,000 per c.mm. respectively. It may be mentioned in this connection that the selection of rabbits from the standpoint of freedom of disease or of age or sex did not appear to have been specifically considered. It is of additional interest, therefore, that the results obtained differ so little from those of the present experiments in which these factors were controlled.

It has been stated above that counts of less than 175,000 or more than 875,000 per c.mm. should be considered definitely abnormal. It should be pointed out, however, that the rabbit in which an abnormal platelet value occurs need not necessarily be an abnormal animal. The classification of abnormality must obviously be made on more inclusive grounds.

#### SUMMARY AND CONCLUSIONS

1. Repeated platelet counts, 991 in number, were made on 148 adult male rabbits of various breeds and types which were specifically selected on the basis of physical fitness and continued freedom from disease. The observations extended over a  $2\frac{1}{2}$  year period (1929-32).
2. A unimodal, peaked, moderately asymmetrical distribution curve was obtained and an analysis of the results gave the following values: the mean, 532,645 platelets per c.mm.; the median, 520,604 per c.mm.; the mode, 503,962 platelets per c.mm. respectively.
3. The extent of the variation among the counts is shown by the standard deviation of 124,960. High platelet counts were found to occur more frequently than low counts, a result that is reflected in a significant skew to the right in the frequency curve.

4. It was calculated that in healthy male rabbits a platelet count of less than 220,000 or more than 845,000 per c.mm. should be considered abnormal.

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THE TRANSMISSION OF NEUROTROPIC YELLOW FEVER  
VIRUS BY STEGOMYIA MOSQUITOES\*BY NELSON C. DAVIS, M.D., WRAY LLOYD, M.D., AND MARTIN  
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Studies on transmission of neurotropic yellow fever virus by mosquitoes have a bearing on the practical problem of vaccination against yellow fever by the method of Sawyer, Kitchen, and Lloyd (1), and on the more theoretical question of reversion of neurotropic virus to the viscerotropic type.

Dinger (2) has reported the production of typical visceral lesions in monkeys by mosquitoes which carried a strain of neurotropic virus. The insects had been induced to feed on a mixture of infected mouse brains and normal blood. However, the strain of virus had been adapted to mice quite recently (tenth to twelfth passages). We are convinced that the longer established French strain (Theiler (3)) acts somewhat differently in mosquitoes.

The present method of vaccination (1) calls for the injection of a small dose of living neurotropic virus, and for the simultaneous administration of large amounts of immune serum. Considering the small dosage of virus used and the difficulty with which mosquitoes acquire infectivity with the strain, it is inconceivable that vaccinated persons might become a menace to their fellows even in the absence of isolation and protection by screens.

On Nov. 22, 1931, F.L.S. was vaccinated by the usual procedure. 15 hours later he was fed upon by more than 150 stegomyia mosquitoes (Lot 669). On Dec. 22, 1931, *M. rhesus* B10 was inoculated intracerebrally with 1 cc. of foreign serum (as a mild irritant) and was fed upon by the mosquitoes immediately after-

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656	<i>M. rhesus</i> A7	+	*	36	Bites	B2	-	-	69	R760	Ic.	†	Source animal belonged to intraspinal neurotropic series
659, 660	Mouse Groups R433, 434	+	+	34	Bites	B7	-	-	60	R761	Ic.	†	Mosquito lots fed on mice at 5 hrs. and at 22 hrs., after large ip. injections of virus
675, 676	Mouse Groups R547, 548	+	+	14	Bites	B5	-	-	14	R629-637	Ic.	?	Lot 675 fed on mice 2 hrs. after ip. injection. Emulsion at once of this lot killed mice in dilution 1:10,000 based on amount of blood ingested
678, 679	<i>M. rhesus</i> B1	+	?	14 44	Bites Injection	B9 C9	- +	- +	44	R791	Ic.	+	Source animal inoculated with large amounts of mouse brain virus on 2 successive days. Mosquitoes fed 24 hrs. after last injection
684	Mouse Groups R605, 606	+	+	29	Injection	C3	-	†	15 29	E267 R761	Ic. Ic.	- †	Mosquitoes containing fresh blood of killed mice, in dilutions of 1:100 and probably higher. First subsequent mouse test made with approximately 2 per cent suspension filtered
695	Mouse Groups R718, 719	+	+	37	Bites	D3	+	+	2 37	6 groups R867	Ic. Ic.	? +	Dilutions of mosquitoes on day of infective blood meal, based on amount of blood ingested, killed up to dilution 1:2,500. Some animals died in tests 2 days later. Virus probably present, but not satisfactorily proved. Proved after 37 days

+ = positive. - = negative. ? = doubtful; or infection not satisfactorily proved. Ip. = intraperitoneally. Ic. = intracerebrally.  
 Subcut. = subcutaneously; subcutaneous injections were made into suckling mice.  
 \* No test made.  
 † Animals died from secondary infections.

ward. The same insects fed upon this animal on Dec. 26, 29, and on Jan. 2, 1932. No temperature reaction developed, and the monkey's serum later proved to have no protective power against yellow fever virus in mice.

In Oct., 1930, two mosquito transmission experiments were performed, one with Asibi strain virus adapted to mice (22nd passage), and one with French strain virus (100th passage in mice). In each experiment a heavy suspension of infected mouse brains was injected into a monkey (*M. rhesus* S1 and *M. rhesus* S2); both animals showed fever on the next day; and mosquitoes were allowed to feed at approximately 24 hours following the inoculation of virus. Neither monkey died of yellow fever. After suitable incubation periods the mosquito lots were permitted to feed on normal monkeys, and the latter subsequently developed fever. The temperature of the animal (*M. rhesus* S3) which received Asibi strain virus through mosquitoes reached 105.4°F. on the 5th day; the temperature of the one (*M. rhesus* S4) which received French strain virus reached a maximum of 104.3°F. on the 14th day after exposure to mosquito bites. Both animals were afterward given test doses of virus and were found to be immune; preceding the immunity test, the serum of *M. rhesus* S4 (which had received French strain virus) gave a strongly positive complement fixation reaction with yellow fever liver antigen.

The experiments listed in Table I were performed for the most part during the last quarter of 1931 and early in 1932. In every case the French neurotropic strain of virus was employed, in passages from the 149th to the 181st. These experiments represent by no means all the attempts made to transmit the neurotropic virus through mosquitoes.

Some difficulty was experienced in determining the most favorable period after the inoculation of virus for the mosquitoes to feed. Monkeys inoculated by the subcutaneous or by the intraperitoneal route with infected mouse brains do not invariably develop fever. Animals which do become febrile following inoculation by these routes, or by either the intracerebral or the intraspinal route as well, do not always have virus in the blood stream at the time of fever. Consequently at first many mosquito lots had to be discarded because the blood of the host was non-infective at the time of feeding. In later experiments mosquitoes were allowed to feed on monkeys at 24 to 48 hours after intraperitoneal or subcutaneous inoculation, and on mice at 2 to 24 hours after intraperitoneal inoculation of virus.

Mosquitoes did not feed well either on artificial mixtures of infected mouse brains and normal defibrinated blood, or on infectious blood *in vitro*, with or without the addition of glucose. Several methods were tried of attracting the insects and inducing them to feed, but none of them ever became fully engorged.

In the single experiment included in Table I in which artificial feeding was employed, the insects of Lot 648 fed on an infected brain-normal blood mixture through the shaven and scraped skin of a recently killed guinea pig. During the time of feeding the lot was divided; some of the insects were kept at incubator temperature (37°C.), and the others were left at room temperature (27°C.). At neither temperature was feeding satisfactory, but in the cage kept at 27°C. more mosquitoes were found afterwards with visible blood in their abdomens.

From the combined lot thirteen insects, which contained recently ingested infective material, were ground up in 3 cc. of 10 per cent normal monkey serum; of this suspension, 0.25 cc. was injected subcutaneously into each of six suckling mice. Three of the latter died within 48 hours; one was dead and two were sick on the 6th day. Transfer of brain material from the sick animals brought down the sub-inoculated groups with yellow fever on the 4th day. 22 days later (Nov. 11) the remaining mosquitoes in Lot 648 were allowed to feed on *M. rhesus* A6. On Nov. 18 and 19 the monkey had a slight fever. On Nov. 23 his temperature suddenly rose to 106°F., and it remained above 105°F. during Nov. 24 and 25. On Nov. 26 the temperature dropped to 100°F. The animal was discovered lying down—weak, but excitable. There was present marked tremor of arms and head, occasional nystagmus, well developed wrist drop, some muscular incoordination, and difficulty in walking. These signs remained salient for 4 days, and never entirely disappeared. Blood taken 7 days after the last fever gave perfect protection against virus in mice. The temperature of the animal continued almost constantly subnormal, and weakness became progressively more marked. On the 10th day after the last fever the monkey was killed when moribund.

As judged by the symptomatology of *rhesus* monkeys inoculated intracerebrally with neurotropic virus (4), the syndrome presented by this monkey immediately after fever was that of acute encephalitis. It was conjectured that the virus localized in the brain because of irritation resulting from an old depressed fracture in the right frontal region of the cranium. However, this old injury may have played no part in the etiology of the acute infection. At the time of death encephalitis had somewhat subsided clinically. Nevertheless upon microscopical examination a few vessels with cuffing were found in Ammon's horn and in the brain stem; necrotic ganglion cells were noted in the medulla oblongata.

It seems unnecessary to describe in detail the other experiments mentioned in Table I. The table, with its observations and footnotes, is self-explanatory. Although circulating virus was present in every source animal at the time of mosquito feeding, and was sometimes verified in the freshly fed mosquitoes, it was not always proved to be

TABLE II

Serial Transmission of Neurotropic Yellow Fever Virus in *M. rhesus* by Means of *Stegomyia Mosquitoes*

Passage of mouse virus in monkeys	Test monkey No.	Animal from which transfer was made	Manner of virus transfer	Mosquito lot used for transfer	Fever in test monkey	Serum infective for mice	Nervous manifestations	Death of test monkey	Encephalitis at death	Brain infective for mice	Subsequent immunity	Mosquito lots fed on test	Day of experiment when fed	Infectivity of mosquitoes for mice																
														Injection immediately after original blood meal			Later test of mosquitoes													
I	A9	Mice (brains) Passage F167	Brain emulsion subcut.	*	-	+	-	-	-	*	+	661	1 (23 hrs.)	Mouse groups	Route of infection	Infection proved	Interval after meal	Mouse groups	Route of infection	Infection proved										
														447	Subcut.	-	65	792	Ic.	-										
														448	Ic.	-	days	792	Ic.	-										
														455	Ic.	+	64	792	Ic.	+										
II	A10	A9	Serum ip.	*	+	-(8th day)	-	-	-	*	+	665	5	477	Ic.	+	*	*	Bites	+										
														456	Subcut.	-	*	*	*	-										
														667	4 and 6	*	*	*	*	*	*	*	*							
														688	4 and 6	*	*	*	*	*	*	*	*							
	B4	A9	Mosquito bites	663	-	*	-	-	-	-	-	686	3 and 5	*	*	*	*	*	*	*										
																					661	Mosquito bites	-	+	+	+	+	+	+	+
																					661	Mosquito sus- pension subcut.	-	+	+	+	+	+	+	+
																					663	Mosquito sus- pension subcut.	-	+	+	+	+	+	+	+



TABLE II—Concluded

Passage of mouse virus in monkeys	Test monkey No.	Animal from which transfer was made	Manner of virus transfer	Mosquito lot used for transfer	Fever in test monkey	Serum infective for mice	Nervous manifestations	Death of test monkey	Encephalitis at death	Brain infective for mice	Subsequent immunity	Mosquito lots fed on test monkey	Day of experiment when fed	Infectivity of mosquitoes for mice							
														Injection immediately after original blood meal			Later test of mosquitoes				
VI	D4	C7	Mosquito bites	702		*				*		*	*	Mouse groups	Route of infection	Infection proved	Interval after original blood meal <i>days</i>	Mouse groups	Route of infection	Infection proved	
	D6	C2 C4 C7	Mosquito sus- pension subcut.	694 699 702		*				*	+	717	4, 8	*	*	*	*	*	*	*	
VII	B6	C10	Mosquito bites	703-704	+	— (11th day)				*		*	*	*	*	*	*	*	*	*	*
	D7	D6	Blood ip.	*	+	*				*		718	2	*	*	*	*	*	*	*	
	D8	D6	Mosquito bites	717	—	*				*		*	*	*	*	*	*	*	*	*	*

\*No test made.

present in the mosquitoes at later dates. Many animals died from secondary infections following injection of mosquitoes, before yellow fever had time to develop.

The suspensions of mosquitoes used for intracerebral inoculation of mice in the experimental work listed in Tables I and II were dilutions of 1:20 to 1:50 in 10 per cent normal monkey (or normal human) serum, centrifuged but not filtered, unless otherwise stated under Observations.

In Table II is summarized an attempt to maintain mouse virus in *rhesus* monkeys, with intermediate passages through mosquitoes.

On Nov. 13, 1931, *M. rhesus* A9 was inoculated subcutaneously with 5 cc. of a 10 per cent suspension of infective brains, from the 167th passage of the French strain of yellow fever virus in mice. Serum from this monkey was injected intracerebrally into mice daily for 10 days following introduction of virus. That taken at 23 hours and at 46 hours proved to be infective; later injections of serum caused no deaths among the mice. Suspensions of freshly fed mosquitoes of the 2nd day and of the 5th day were infective for mice. The mosquitoes of the 2nd day later failed to transmit yellow fever; those of the 5th day were not tested further. Sera of the 1st and 2nd days (Nov. 14 and 15) were injected intraperitoneally into *M. rhesus* A10. The latter developed a high fever (maximum temperature 105.8°F.) on the 9th to 11th days after the injection; *M. rhesus* A9 never had a fever.

Mosquito Lot 667<sup>1</sup> was allowed to feed on *M. rhesus* A10 on Nov. 18 and 20, before the development of fever. The blood taken on Nov. 23 (1st day of fever) was non-infective for mice and gave a positive protection test against yellow fever virus. On Dec. 19 mosquito Lot 667 fed on *M. rhesus* B8. On the 4th day afterward fresh mosquitoes (Lot 690) engorged on the same monkey. On the 7th day (Dec. 26) the animal had a temperature of 105.4°F. Mosquito Lot 690 was allowed to feed once more. Blood transferred to *M. rhesus* B11 at this time caused no reaction, although the recipient was immunized by the injection. On Dec. 28 (3rd day of fever) *M. rhesus* B8 showed very definite neurological signs. Symptomatology included: paresis of arms, more marked on right; wrist drop; weakness; tremor of head and limbs, most marked in right arm; occasional nystagmus; incoordination, with difficulty in walking and in righting himself; crossing of arms when in sitting posture; hiding of head against side of cage or on floor; loss of fight; sharp, shrill cries. In the afternoon of the same day the temperature was found

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<sup>1</sup> As a routine, female *stegomyia* mosquitoes are placed 180 to 200 to a cage. When 1 to 2 weeks old nearly all of them engorge when a monkey is introduced directly within their cage. It is safe to say that a batch or lot of freshly fed mosquitoes after the removal of the non-engorged and after due allowance for deaths since original counting and separation, consists invariably of well over 150 insects.



## TRANSMISSION OF NEUROTROPIC YELLOW FEVER

to be falling; tremor was accentuated; when placed on his feet the animal swayed, fell, and recovered with difficulty. In the certainty that death would occur during the night, the animal was killed with chloroform.

At autopsy *M. rhesus* B8 showed very few gross lesions. There was atrophy of subcutaneous fat, the spleen was slightly enlarged, and the liver was paler than normal; otherwise the organs were negative. Microscopically the liver showed infiltration of fat, particularly around the portal spaces; there appeared to be a slight parenchymatous degeneration, but no necrosis; no intranuclear inclusions were found. The kidney showed a slight cloudy swelling, with a few casts in the medulla. The spleen revealed occasional small necroses in the germinal centers of the follicles. Sections from the brain were of the greatest interest. There was no meningeal reaction. Inflammation was widely distributed throughout the brain, but the cerebellum was almost entirely spared. Cuffing of vessels was more marked in the brain stem. Tiny inflammatory foci and diffuse infiltration of leucocytes were noted in cerebral cortex, hippocampus, and pons. In some fields polymorphonuclear neutrophils were prominent in addition to round cells. Degenerated and necrotic nerve cells were present. There was a relative or actual increase in glial cells. No intranuclear inclusions were found. The picture was that of a disseminated encephalomyelitis, fully as well developed as that produced by Lloyd and Penna (4), or as that produced by Sellards (5) and described by Goodpasture (6), in monkeys inoculated intracerebrally with neurotropic yellow fever virus.

Intracerebral inoculation of mice with brain substance from *M. rhesus* B8 killed two groups (twelve animals) in 4 to 6 days. Transfer of brain material from these to other groups brought down eleven out of twelve animals in 4 days. Thus the infection behaved like that produced by fixed neurotropic yellow fever virus. A protection test performed with this strain was unfortunately complicated by mouse typhoid. However, taking the six day reading as final, the result was as follows: Three groups (eighteen mice) given suspected virus emulsion and yellow fever immune serum showed one death on the 5th day, but no other animals sick up to the 6th day; two groups (nine mice surviving initial inoculation) given suspected virus and normal serum showed six dead and three sick; that is, all either dead or sick, on the 6th day.

Brain emulsion from *M. rhesus* B8 injected intracerebrally into *M. rhesus* C1 caused fever on the 3rd day. On the 7th day (Jan. 4, 1932) the animal had a falling temperature and fully developed clinical manifestations of encephalitis, in every way comparable to those produced by neurotropic yellow fever virus in the experiments of Lloyd and Penna at this laboratory. In the evening of the same day the monkey was obviously moribund and was killed with chloroform. Brain sections from this monkey showed a typical encephalomyelitis. The virus from the brain of *M. rhesus* C1 behaved in mice as the fixed neurotropic strain of yellow fever virus (mouse Groups R713 and R737).

On Jan. 16 mosquito Lot 690, which had obtained its infective blood meal on *M. rhesus* B8, fed on *M. rhesus* C5. 2 days later the monkey showed a tempera-

ture of 104°F. The blood at that time was infective for mice, killing all six of Group R823. On the 3rd day the temperature of *M. rhesus* C5 reached 105.8°F. There was a remission on the 6th day followed by fever until the 10th day, when the temperature again fell below 104°F. On the afternoon of that day the monkey was very weak, and evidently moribund; there were no definite neurological signs. Autopsy showed diffuse tuberculosis. Brain substance from this animal, both that filtered through Berkefeld N and that which was unfiltered, proved fully virulent to mice (Groups R829 and R830). The virus acted in all respects like the fixed neurotropic strain of yellow fever virus. Sections from the brain of *M. rhesus* C5 did not show as typical an encephalitis as those from Monkeys B8 and C1. There was a little hemorrhage into the pia-arachnoid over the medulla; a few vessels in the pons showed perivascular infiltration.

In the series an autopsy was performed upon but one other monkey, *M. rhesus* C7. This animal had received serum intraperitoneally from *M. rhesus* C5. Fever developed on the 9th day, at which time he was bound and put into a cage for mosquito feeding. While there he collapsed. He was removed in a moribund condition. The brain showed no perivascular infiltration, but occasional ganglion cells were degenerated (nuclear fading; cytolysis). Serum from *M. rhesus* C7 was non-infective for mice. However, brain emulsion filtered through Berkefeld candle V killed three out of six mice (Group R831) inoculated intracerebrally; subinoculated mice were killed promptly on the 4th day, precisely as with fixed neurotropic yellow fever virus.

In the four animals studied at autopsy neither the macroscopic nor the microscopic lesions were typical of those caused by viscerotropic yellow fever virus. In none of the animals did the liver show necrosis.

It was thought upon starting the experiment that repeated passage through mosquitoes might cause a reversion of the virus from the neurotropic to the viscerotropic type. Such a reversion was not demonstrated. Apparently the virus became progressively weaker and died out in the sixth passage from mice. It is quite possible that mosquitoes were not allowed to feed at the right time for picking up the maximum amount of virus. At the time of fever the blood of several monkeys was shown to be non-infective. Since it was impossible to rely upon fever as a guide, the time for feeding mosquitoes had to be chosen quite arbitrarily.

In Table I it will be noted that mosquitoes became infected by feeding on mice at a proper interval after the inoculation of the latter with large amounts of neurotropic virus. In Table II there is recorded the infection of mice by the bites of mosquito Lot 667, which obtained its infective blood meal from *M. rhesus* A10, 47 days previously. The

mouse group (R712) consisted of six baby mice about 2 weeks old. Three of the six became ill within the usual incubation period. From one showing complete paralysis of the hind legs, brain transfer was made to adult mice of Group R757. All of the latter were stricken on the 4th day, as is usual following intracerebral inoculation of fixed neurotropic virus.

Mosquito feeding on mice was accomplished with surprising ease. The animals were placed in cylinders about 1 inch in diameter, made of strong, wide meshed wire gauze. The ends of the cylinders were packed with cotton and strapped with adhesive plaster. Mosquitoes attacked the mice at once and engorged rapidly.

#### DISCUSSION

Lloyd and Penna (4) have already reported experiments demonstrating the fixed nature of the neurotropic French strain of yellow fever virus. The present experiments are confirmatory of this fundamental change in the nature of the virus. The adaptation to mouse brains does not signify an attenuation; under certain conditions the virus is still lethal for *rhesus* monkeys. However, the blood stream is only transiently invaded; no marked lesions are produced in liver and kidneys; and final localization occurs predominantly or entirely in the nervous system. This is true even after passage through mosquitoes. There may be means of inducing a reversion to the viscerotropic type, but such means have not yet been discovered by us.

#### SUMMARY

1. By the bites of *stegomyia* mosquitoes carrying neurotropic yellow fever virus, encephalitis has been produced both in white mice and in *rhesus* monkeys.
2. The fixed neurotropic strain of virus cannot be maintained in the mosquito host as well as can the viscerotropic strains. This is doubtless attributable in part to a smaller amount of virus ingested, because of paucity in the blood stream of the mammalian host.
3. These experiments furnish additional evidence that the long established neurotropic yellow fever virus has changed fundamentally from the parent French strain.

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## THE CELLULAR REACTIONS TO LIPOID FRACTIONS FROM ACID-FAST BACILLI

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PLATES 44 TO 46

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The biological tests thus far made in this laboratory (1-12) with chemical fractions isolated from or liberated by the growth of tubercle bacilli have been directed toward a clearer understanding of the tissue changes in tuberculosis and the rôle of the various chemical factors in the production of these changes. Sabin (12) and Anderson (13) have recently made critical reviews of the earlier chemical and biological investigations of tubercle bacilli and analyzed the results obtained by the various groups now working under a plan for cooperative research on tuberculosis (14).

The lipid portion of the organisms, notably a phosphatide constituent, possesses the capacity of producing tubercular tissue; that is, epithelioid cells and giant cells (1-5, 9). The so called waxes cause a proliferation of fibroblasts (9). The acetone-soluble fat induces proliferation of all connective tissue cells and of blood vessels, and causes hemorrhage (15). The polysaccharide is chemotactic for and toxic to leucocytes (6, 9). The protein is probably responsible for fever, and in addition causes a proliferation of plasma cells (16).

By means of the recent observations it has been possible to divide the disease, so to speak, into several parts, for each of which a particular chemical fraction of the bacilli is responsible.

Sabin and Doan (2) made cultures from the phosphatide and from the tissues of animals which received the phosphatide, with consistently negative results. Nevertheless, in the present study cultures and stains for acid-fast organisms have again been made to rule out contamination of the phospholipins by living, dead, or partially defatted organisms. Furthermore, a comparison has been made between the reactions produced in the tissues by the heat-killed and partially

defatted organisms and those produced by certain of the lipoids. Comparative observations have been made on the effects produced by introducing into the tissues of animals the phosphatides from five strains of acid-fast bacilli. These were: the human, bovine, and avian tubercle bacilli, the timothy grass bacillus, and a strain of acid-fast bacilli isolated from a leprosy human being. When it was found that each of the bacterial phosphatides produced similar cellular reactions, attention was directed toward the manner in which these changes were brought about. Observations were then made on the fate of the injected lipid and the newly formed tissue produced by it. In this way the complete life cycle of the epithelioid cell has been determined and information gained regarding the physiological properties and potentialities of this strain of connective tissue cells.

### *Material and Methods*

The bacteria for the chemical analyses have been grown in large quantities under standardized conditions by the H. K. Mulford Co. The human tubercle bacillus used was the H-37 (Saranac) strain. The bovine and avian strains were of known virulence. The timothy grass and lepra strains, avirulent for laboratory animals, were obtained from the Hygienic Laboratory, Washington, D. C. This was the name of the laboratory at the time the organisms were obtained. Since June, 1930, it has been known as the National Institute of Health.

The phosphatides isolated by Anderson from human (17), bovine (18), and avian (19) tubercle bacilli, and the timothy grass bacillus (20) are gray-white, amorphous solids. That from the leprosy bacillus (21) is pale lavender in color. These substances are soluble in alcohol and ether, insoluble in acetone, and may be readily mixed with water to form milky suspensions free from flocculi or sediment. The first preparations of phosphatides received from Dr. Anderson contained traces of acid-fast debris (2). More recently the ether solution of the lipid has been passed twice through Chamberland candles and none of the preparations contains demonstrable acid-fast material. The phosphatide from the human tubercle bacillus has been examined under crossed Nicol prisms by Dr. R. W. G. Wyckoff of The Rockefeller Institute, who pronounced it predominantly crystalline. Twenty-two plates of Petroff's egg-gentian violet media, each seeded with 10 mg. of one of the phosphatides in aqueous suspension, have remained free of acid-fast bacteria after 4 months' incubation. Guillery (22) has likewise failed to find viable organisms in the phosphatide.

Rabbits and guinea pigs have been used in the experiments. The phosphatides were introduced by the intraperitoneal, subcutaneous, and intrapulmonary routes. The material was injected into the lungs through the chest wall and by way of the trachea. The intraperitoneal route is the one of choice for determining the type

of cell affected by the foreign material. The omentum participates most actively in the reaction and is well adapted for study either of fresh tissue or fixed material. The subcutaneous route is better for determining the quantitative reaction. The reaction in the lung offers a comparison with tuberculosis itself, and an opportunity to study the effects in a site where there is a minimal amount of connective tissue.

For the intraperitoneal route the usual dose of the phosphatide was 80 mg. in aqueous suspension. The reaction has been studied after from one to thirteen injections. In some instances a single massive dose was given and the tissues were studied about 2 weeks later. Each animal receiving the divided doses was killed by intravenous injection of air 24 hours after the last injection. Fresh tissues were studied by the supravital method. Blocks of tissue were fixed in Helly's mixture, embedded in paraffin, sectioned, and stained with hematoxylin and eosin, and by Giemsa's method.

### *The Phosphatide from Human Tubercle Bacilli*

*A. Evidence That the Phosphatide Is Phagocytized.*—If a small granule of the phosphatide be placed on a slide with a drop of water over which is a thin cover-slip, the material first has a granular appearance under the microscope. However, within a few minutes the edges of the lipoid advance into the liquid and take the appearance of degenerating myelin. If this procedure be repeated, using a slide on which is a thin film of neutral red, the myelin figures take a faint pink stain. This staining reaction of the free phosphatide does not change on standing overnight.

If a single layer of the fresh omentum of a normal rabbit which has received an intraperitoneal injection of the phospholipin be studied on an unstained slide within 12 hours after this injection, the cells of the milk spots will be seen to contain these same myelin figures. They are contained in large vacuoles of irregular size and shape. Stained with neutral red, the myelin figures in many of the cells show the same staining reaction as the phosphatide alone. However, if the examination be made at a later period, or if these cells with unaltered staining of the phosphatide be studied after a few hours, the material in the cytoplasm becomes a deeper red. Fig. 1 shows one of these large cells containing the myelin figures. This photograph was made from a supravital preparation of peritoneal exudate of Rabbit R 1802,<sup>1</sup> which had three doses of the phosphatide and was killed 24 hours after the last dose. Note the large, irregular masses in the cytoplasm and that they all are of equal density. Fig. 2 illustrates the appearance of these cells in fixed preparations. In the tissues of the guinea pig these cells may be seen 24 hours or more after the phosphatide has been injected. Fig. 13 shows two cells from the supravital preparation of peritoneal exudate from Guinea Pig R 690. Note the appearance of degenerating myelin. The smaller of the two cells shows the phagocytized material in more finely divided form. Similar cells from fixed preparations of the omentum of the same animal are shown in Fig. 14.

<sup>1</sup> These are serial numbers of animals used in this laboratory during a period of years.



It is clear from these results that the cells of the milk spots phagocytize the phosphatide. It will be shown that the lipoid undergoes a definite type of degradation within the cells, and that the cells which accomplish this process are derived from monocytes or their stem cells in the connective tissues.

Everywhere in the connective tissues of the body are tiny foci of young, undifferentiated connective tissue cells and monocytes. In the omentum these foci are called the milk spots. In the interspaces between the milk spots of the omentum are the fibroblasts and clasmatocytes or macrophages—the latter probably always functioning with reference to substances passing through the omentum. It is the cells of the milk spots, however, which exhibit the specific response to tuberculo-phosphatide (see Sabin, Doan, and Forkner (9, Fig. 4)).

Rabbit R 2253 received two injections of 80 mg. each of the phosphatide from human tubercle bacilli intraperitoneally at 24 hour intervals. The animal was killed 2 days following the second injection. The peritoneal fluid was slightly cloudy, quite cellular, and showed 75 per cent of cells of the monocyte series; that is, monocytes, stimulated monocytes, and epithelioid cells. The omentum was considerably thickened and there was an increase in both the number and size of milk spots. In the supravital and unstained preparations of the omentum, peritoneal fluid, and retrosternal lymph nodes small amounts of the phosphatide could be seen free in the intercellular spaces. This lipoid looked like degenerating myelin, just as in the study of the phosphatide itself. In addition, numerous cells were seen, particularly in the retrosternal lymph nodes, which were filled with the myelin figures: apparently unchanged phosphatide. In sections, the cytoplasm of these cells was filled with clear spaces of varying size and shape. (Similar cells are shown in Figs. 1 and 2.) The nuclei contained one to three nucleoli. The cytoplasm was quite basophilic, and in many of the cells numerous mitochondria were seen. The cells of the omentum containing the myelin figures were confined almost wholly to the milk spots. However, many, if not the majority of the cells of the milk spots, and those in the retrosternal nodes contained vacuoles of reduced, uniform size, all of which stained the same color with neutral red. Cells similar in appearance but from other animals can be seen in Figs. 3 and 4. In the latter the process of intracellular dispersion of the lipoid into finer and finer particles had already begun.

Rabbit R 1802 received three daily injections of 80 mg. each of the H-37 phosphatide intraperitoneally and was killed 24 hours after the last dose. The milk spots of the omentum were markedly stimulated and there were many epithelioid cells in the interspaces which had become diffusely involved. There were moderate numbers of active granulocytes in the supravital preparations of the peritoneal

fluid and in the omentum. All transitions from the most primitive mesenchymal cell to the typical fine vacuole epithelioid cell could be seen in the omentum, although the latter were not numerous. Many cells with large vacuoles of irregular size and shape (Fig. 1), all of which took the same stain with neutral red, were present in the omentum and peritoneal fluid. Again the phagocytized material, tuberculo-phosphatide, had the appearance of degenerating myelin.

Guinea Pig R 2254 received three injections of 40 mg. each of the H-37 phosphatide intraperitoneally at 24 hour intervals and was killed 4 hours following the last dose. The omentum was enormously thickened. The milk spots were large and increased in number. Numerous small, white flakes appeared on the visceral and parietal peritoneum. The peritoneal fluid was thick. The unstained spread of the omentum showed that the milk spots were bright and refractile while the interspaces were duller. The large phagocytic cells in the milk spots showed definite myelin-like figures in the cytoplasm. (Similar cells are shown in Figs. 13 and 14.) In the supravital preparations some of these myelin-like masses were pale pink, while in other cells the vacuoles were still of the same irregular shape and size but stained more deeply. After 2 hours at room temperature many of the pale cells became more deeply stained, even though the cells were obviously still alive. These cells, derived from monocytes, which contain myelin-like masses in large vacuoles of irregular size and shape but uniform staining reaction, will henceforth be called first stage epithelioid cells.

*B. Evidence That the Phosphatide Is Degraded within Cells.*—24 hours after a single dose of the phosphatide from the human tubercle bacillus, the milk spots of the omentum appeared enlarged: when examined microscopically it could be seen that the young connective tissue cells and monocytes had phagocytized the phosphatide in large quantities (Rabbits R 819, R 2122, and R 2185 in Table I). The lipid was in large vacuoles of irregular size and shape (Figs. 1 and 2). The staining reaction with neutral red was uniform, slightly toward the acid range of this dye (9, Figs. 1 and 2). At this time the increase in size of the milk spots was caused by the increased size of the cells which had phagocytized the lipid. A few of the cells of the interspaces contained a single vacuole of the lipid, but in general they did not participate actively in the reaction. Moderate numbers of neutrophilic leucocytes responded to the first but not to subsequent injections, and those which appeared with the first injection were soon phagocytized by clasmotocytes and removed. These leucocytes exhibited no evidence of phagocytic activity for the phosphatide.

24 hours after two daily injections of the phosphatide, the milk spots of the omentum were still larger. The young, undifferentiated connective tissue cells showed evidence of rapid maturation toward monocytes, although the cytoplasm was filled with the large, irregular shaped vacuoles. Numerous mitotic figures were present (9, Fig. 3). In sections the cells of the milk spots were as large as epithelioid cells and their cytoplasm was filled with large, clear spaces from which the lipid had been removed during fixation (R 820, Table I).

After three daily injections of the phosphatide, the omentum was markedly

thickened, due to the increased size and number of milk spots (R 821, Table I). Many mitoses were seen, as well as cells dividing by amitosis. In spreads or scrapings of the omentum another significant change was noted. More of the cells of the milk spots had vacuoles of uniform size but considerably smaller than in the earlier stages. The staining reaction to neutral red was unchanged. The cytoplasm of these cells appeared foamy in sections.

Fig. 3 shows a second stage epithelioid cell from the supravital preparation of peritoneal exudate from Rabbit R 2186. Note that the cytoplasm is filled with vacuoles of uniform size and shape, but considerably smaller than those of the cell in Fig. 1. Fig. 4 is from the fixed section of omentum of the same animal as represented in Fig. 3. Figs. 3 and 4 then show the second stage epithelioid cell as it appears in the two techniques. It will be seen that this cell becomes the typical epithelioid cell, indistinguishable from those occurring in the disease. Two stages in the life cycle of this cell may now be recognized: the first, in which the vacuoles are very large but of irregular size and shape; and the second, in which the vacuoles are smaller and of uniform size.

During the 2nd week after a single large dose, or after repeated daily doses of the phosphatide, the omentum appeared massively thickened (R 2409 and R 2408, Table I). The identity of the individual milk spots was lost in the hyperplastic mass of newly formed tissue. There was moderate hyperemia of the vessels. Scattered here and there over the intestinal serosa and parietal peritoneum were small, white nodules, not firmly adherent. Some of the new tissue could be seen on the surface of all the abdominal viscera. By this time the omentum was usually too thick for study of spreads by the supravital method. Scrapings of the omentum and other organs showed the predominating cell to be the typical epithelioid cell with rosette of fine vacuoles staining uniformly (9, Fig. 8). In sections the cytoplasm of these cells appeared homogeneous (Figs. 5 and 6). The epithelioid cells were in many places grouped together, forming tubercles closely resembling those seen in the disease. Numerous typical rosette giant cells were present. By a comparison of Figs. 1 and 2, representing first stage epithelioid cells, with Figs. 3 and 4, representing the second stage epithelioid cells, and with Figs. 5 and 6, representing the third stage cells, the gradual process of intracellular dispersion of the lipid into more numerous and finer particles can be visualized.

At this stage, but never in the early reaction, rather numerous lymphocytes and smaller numbers of plasma cells were present. Both the lymphocytes and plasma cells tended to occur in clumps about the tubercle-like structures, and especially about clumps of Langhans giant cells, but the plasma cells remained apart and did not become scattered between the lymphocytes.

After the lipid had been dispersed into the finest vacuoles no further perceptible change took place. The epithelioid cells so produced were remarkably persistent (R 377 and R 379, Table I); small clumps of these cells have been found 280 days after a single intrapulmonary injection of 50 mg. of the phosphatide (R 1644, Table III). However, gradual resorption of the new tissue did take place. The areas of more diffuse reaction disappeared first, the tubercles later (R 368, R 377,

TABLE I

*Protocols of Rabbits Which Received Phosphatide from Human Tubercle Bacilli Intraperitoneally*

Animal No.	No. of injections	Amount of each injection	Interval between last injection and autopsy	Observations
		mg.	days	
R 819	1	80	1	Milk spots of omentum larger than normal. Many primitive cells, monocytes, stimulated monocytes, and phagocytic cells with large, irregular vacuoles staining uniformly. Large numbers of granulocytes, free and in clasmatocytes
R 2122	1	80	1	Same as preceding animal (R 819). In addition, many monocytes and first stage epithelioid cells in retrosternal lymph nodes
R 2185	1	80	1	Same as preceding animal (R 2122)
R 820	2	80	1	Definite accentuation of milk spots of omentum. They were made up principally of primitive cells, monocytes, and first stage epithelioid cells, with a few second stage. Numerous polymorphonuclears and clasmatocytes. Considerable cell division
R 821	3	80	1	Milk spots increased in size and number. Considerable cell division. Few polymorphonuclears and clasmatocytes. Many first, a few second, and a very few third stage epithelioid cells. An occasional rosette giant cell and a few small areas of caseation
R 2123	1 1	100 100*	6	Milk spots markedly increased in size and number. Middle lobe right lung partly consolidated. Pleural and peritoneal fluids turbid due to many monocytes and second stage epithelioid cells. In the omentum and lung the cells, chiefly second and third stage epithelioid cells, were grouped into tubercle-like masses around which were numerous lymphocytes. Epithelioid cells and caseation in retrosternal lymph nodes
R 2186	10	80	1	Entire omentum greatly thickened. Abdominal viscera covered with thin film of exudate (epithelioid cells and monocytes). Massive diffuse and nodular involvement of omentum and retrosternal nodes with principally third stage epithelioid cells. Numerous lymphocytes, a few giant cells and plasma cells. Moderate amount of caseation
R 2409	10	80	2	Same as R 2186, except perhaps greater lymphocytic response
R 664	11	80	1	Massive thickening of omentum. Many tubercle-like masses of third stage epithelioid cells. Many rosette giant cells. Many plasma cells and lymphocytes. Moderate amount of caseation
R 1016	1	1000	11	Omentum massively thickened due to tubercle-like masses and diffusely scattered epithelioid cells. Massive involvement of retrosternal nodes with typical epithelioid cells. Predominating cell in omentum was the third stage epithelioid cell. Many lymphocytes, a few plasma and giant cells. Moderate amount of caseation
R 2408	1	1000	14	Same observations as in R 1016, except few lymphocytes and many plasma cells and Russell body cells about the tubercle-like masses of epithelioid and giant cells

\* This injection was made intrapleurally at the same time as the intraperitoneal injection.

TABLE I—*Concluded*

Animal No.	No. of injections	Amount of each injection	Interval between last injection and autopsy	Observations
		<i>mg.</i>	<i>days</i>	
R 2187	10	80	7	Reaction as in R 2408, but more caseation. Extensive involvement of retrosternal and tracheal lymph nodes
R 368	13	80	30	Omentum massively thickened and bound to intestine by adhesions. Nodular masses of third stage epithelioid cells and giant cells. Many plasma cells and lymphocytes. A little caseation
R 377	13	80	90	Omentum only moderately thickened. Microscopically all the diffuse reaction had disappeared. A few masses of third stage epithelioid and giant cells without caseation or plasma cells but with a few lymphocytes. Numerous degenerating epithelioid cells
R 379	13	80	134	Omentum slightly thickened. Reaction similar to R 377 but only about one-half as extensive. Many degenerating epithelioid cells. A few epithelioid cells remained in the retrosternal nodes

and R 379, Table I). As Sabin, Doan, and Forkner (9) have pointed out, this resorption is accomplished in part by caseation, and in part by a process of resolution similar to that by which experimental tuberculosis of bone marrow regresses (25). It is in this late phase of the cellular reaction to tuberculo-phosphatide that the end stage in the life cycle of the epithelioid cell is to be seen.

Rabbits R 377 and R 379 (Table I) showed only discrete tubercle-like masses of epithelioid cells in the omentum, with an occasional giant cell. All the diffuse reaction had disappeared. There were no lymphocytes or plasma cells. Many of the epithelioid cells in the tubercle-like structures had pyknotic nuclei. In other instances the nuclei were fragmented, yet stained densely. In still other instances the nuclei were paler than normal and presented irregular fringed margins (Fig. 5). These cells with pyknotic or pale nuclei had irregular cytoplasmic borders. Large, irregular, clear spaces could be seen in the cytoplasm. They were obviously dead and disintegrating. The life cycle of this cell has now been completed. It has been seen to arise from a primitive cell (4) in the connective tissues (reticular or mesenchymal cell), to pass through the monocyte phase to that of the epithelioid cell. In the latter phase, four distinct stages have been observed; namely, that with large, irregular vacuoles, that with vacuoles of intermediate but uniform size, that with the smallest, dust-like vacuoles, and the stage of degeneration.

In every animal receiving the phosphatide there has been some caseation (Fig. 9). No area has been designated caseous unless epithelioid cells could be seen within, or at the margin of, the caseous mass. Less caseation has been seen after the phosphatide from the human tubercle bacillus than after the other phospholipins. Further studies of the mechanisms by which caseation is produced are in progress.

*The Phosphatide from the Avian Tubercle Bacillus*

This lipid (13) represents 2.26 per cent by weight of the organisms. It has been studied in the same manner as the phosphatide from the human tubercle bacillus. In the tissues of the normal rabbit the reaction to the avian phosphatide (Fig. 7) most closely paralleled that produced by the phosphatide from the H-37 strain of bacilli.

The avian phosphatide was phagocytized by the cells of the milk spots and remained in large vacuoles (R 686 and R 687, Table II) of irregular shape and size for about 5 days. However, a few of these epithelioid cells of the first stage could be seen at the end of 1 week (R 685, Table II). During the 2nd week the greater number of cells in the omentum were of the second stage, with vacuoles of intermediate but uniform size and shape (R 2177, Table II). From the 12th day, greater numbers of the cells with the finest vacuoles were seen (R 684 and R 688, Table II); but the process of intracellular dispersion of the lipid into the finest vacuoles was not completed until the 3rd week. It is therefore clear that a little more time was required for dispersion into the finest vacuoles than was the case with the phosphatide from the human tubercle bacillus. Also, at any given period the reaction was more mixed. For example, at 7 days (Rabbit R 685, Table II), there remained a few cells of the first stage, while the majority were in the second; and very rarely a cell with the finest vacuoles could be seen.

In addition, the reaction to the avian phosphatide was characterized by greater numbers of plasma cells than were seen after any of the other phosphatides, except that from the bovine organisms. It produced more caseation than any of the other phospholipins except that from the timothy grass bacillus.

*The Phosphatide from the Bovine Tubercle Bacillus*

The phosphatide represents 1.55 per cent by weight of the bovine tubercle bacillus (13). Injected intraperitoneally, it produced a reaction closely resembling those just described (Fig. 8). However, the cells which phagocytized this lipid showed slower and more irregular intracellular dispersion of the lipid than was seen after injections of the phosphatides from human or avian tubercle bacilli.

All three stages of the epithelioid cell could be seen at the 12th day (R 1015 and R 1014, Table III). By the end of the 3rd week all the cells reached the stage with the finest vacuoles (R 1621, Table III). This lipid produced more rosette or Langhans giant cells than any of the other phospholipins. The epithelioid and giant cells occurred both in distinct tubercles and diffusely as in the reaction to the other phosphatides.

## LIPOID FRACTIONS FROM ACID-FAST BACILLI

Greater numbers of plasma cells and lymphocytes also characterized the reaction (R 1144 and R 1145, Table III). In many areas tubercles of epithelioid giant cells were surrounded by a broad ring of lymphocytes and plasma cells.

TABLE II  
*Protocols of Rabbits Which Received Phosphatide from Avian Tubercle Bacillus Intrapеритонеally*

Animal No.	No. of injections	Amount of each injection	Interval between last injection and autopsy	Observations
R 686	1	mg. 80	days 1	Slight stimulation of milk spots of omentum. Many polymorphonuclears and clasmatocytes. Predominating cell was a young connective tissue cell or monocyte, the cytoplasm of which was filled with large vacuoles of irregular shape but uniform staining reaction to neutral red.
R 687	2	80	1	Milk spots of omentum moderately increased in size and number. Tiny white nodules composed of fibrin and monocytes over abdominal viscera. Numerous polymorphonuclears free and in clasmatocytes. Predominating cell in omentum was first stage epithelioid cell. There was a little caseation.
R 685	7	80	1	Marked general thickening of omentum. Microscopically many tubercle-like masses and diffusely scattered epithelioid cells of first and second stage—the latter predominating. Occasional rosette giant cell. Many plasma cells, a few lymphocytes, and a great deal of caseation.
R 2177	10	96	1	Omentum greatly thickened. Identity of milk spots almost lost. Several loosely attached small, yellow-white nodules over abdominal viscera. Many tubercle-like masses in omentum. Epithelioid cells of all stages, second and third predominating. Few polymorphonuclears. Numerous plasma cells and lymphocytes. Extensive reaction in retrosternal nodes: epithelioids and rosette as well as foreign body giant cells. Moderate amount of caseation.
R 684	12	80	1	Reaction closely resembled R 2177 but there were more plasma cells and more epithelioid cells with fine vacuoles.
R 688	12	80	2	Reaction very closely resembled that seen in R 2177.
R 1149	1	1000	14	Massive reaction in omentum and retrosternal lymph nodes. Epithelioid cells of all stages, those of second predominating. Numerous tubercle-like structures. Moderate numbers of giant cells and plasma cells, fewer lymphocytes and a moderate amount of caseation.
R 1150	1	1000	15	Resembled R 1149 in every respect except that there were more rosette giant cells in the omentum.

The phosphatide from the bovine tubercle bacillus produced less caseation than that from the avian but more than that from the human strain of organisms. In all other respects the reaction was identical to that produced by the other phosphatides from mycobacteria.

TABLE III

*Protocols of Rabbits Which Received Phosphatide from Bovine Tubercle Bacilli*

Animal No.	Route of injection	No. of injections	Amount of each injection	Interval between last injection and autopsy	Observations
			mg.	days	
R 1015	Intraperitoneal	11	80	1	Massive reaction in omentum and retrosternal nodes. In omentum, all stages of epithelioid cells, first and second in largest numbers. In retrosternal nodes, second and third stage epithelioids predominated. Many giant cells. Moderate numbers lymphocytes and plasma cells. Moderate amount caseation. Many tubercle-like structures
R 1014	"	12	80	1	Resembled R 1015 in every respect except that reaction was characterized by fewer first and more third stage epithelioid cells
R 1144	Intraperitoneal and subcutaneous	1	1000	13	Reaction resembled that seen in R 1015. There were many plasma cells and lymphocytes. Epithelioid cells with smallest vacuoles most numerous. Massive reaction in subcutaneous tissues
R 1145	" "	1	1000	15	Massive reaction in omentum and retrosternal nodes. Also in subcutaneous region of right groin. A few first and second and many third stage epithelioid cells. Many giant cells, plasma cells, and lymphocytes, the latter about periphery of tubercle-like structures. Moderate amount of caseation
R 1620	Into right pleura and lung	2	20 100	13	The right lung was adherent to pleura at apex. Right upper lobe consolidated. A few first and second, and many third stage epithelioid cells, both in tubercle-like masses and diffusely scattered in alveoli. Many giant cells, plasma cells, and lymphocytes. Rather extensive caseation. Other viscera normal
R 1621	Intrapleural	1	50	23	Moderately extensive reaction on the pleura. A few friable adhesions. Only third stage epithelioid cells. Numerous giant cells. Many lymphocytes about pseudotubercles. No caseation
R 1646	Intratracheal	1	50	24	Several macroscopic areas in lungs resembling tubercles. These were composed principally of second and third stage epithelioid cells, the latter being more numerous. There were many giant cells and a few lymphocytes but no caseation. Other organs normal
R 1647	"	1	50	137	No macroscopic changes. A few tiny tubercle-like masses of third stage (and degenerating) epithelioids with an occasional giant cell and a few lymphocytes were scattered through the lungs. A few epithelioid cells in tracheal lymph node. Other viscera normal



## LIPOID FRACTIONS FROM ACID-FAST BACILLI

TABLE III—*Concluded*

Animal No.	Route of injection	No. of injections	Amount of each injection	Interval between last injection and autopsy	Observations
R 1642	Intrapleural	1	mg. 50	days 139	No pleural adhesions. Small, yellow-pink nodule in upper lobe of right lung. Microscopically there were scattered clumps of third stage (and degenerating) epithelioid and giant cells, surrounded by a few lymphocytes in the right lung. A few epithelioid cells were seen in the tracheal and retrosternal lymph nodes. Other organs normal
R 1645	Intratracheal	1	75	196	Animal died. Had been kept in room with tuberculous animals and had renal tuberculosis. There were scattered small lesions in the lungs with unequivocal second stage epithelioid cells, indicating acquired pulmonary tuberculosis. Unsatisfactory for comparison of reaction or study of regression
R 1644	Intrapleural	1	50	280	All organs appeared normal at autopsy. In sections of the lungs a very few small clumps of epithelioid cells with an occasional giant cell were seen. About the periphery of these were a few lymphocytes. The epithelioid cells contained carbon particles but the giant cells were typical rosette types, interpreted as a last remnant of the reaction

*The Phosphatide from the Timothy Grass Bacillus*

This fraction (13) represents 0.59 per cent by weight of the organisms. It has been studied less intensively than the previously discussed phosphatides. However, it is interesting to note that this fraction, from an ordinarily avirulent strain of acid-fast organisms, produced epithelioid cells and tubercles (Fig. 9) in amounts equivalent to the other phosphatides.

This phosphatide, administered intraperitoneally, was also phagocytized by the milk spots of the omentum. On the 2nd day the predominating cell was the first stage epithelioid cell with the largest vacuoles (R 1427, Table IV). At 14 days a few of these cells remained but there were greater numbers of the cells of the second stage (R 1426, Table IV). Smaller numbers of typical epithelioid cells with rosettes of the finest vacuoles were seen at this stage, together with moderate numbers of rosette giant cells, numerous plasma cells, and a few lymphocytes. There was a great deal of caseation (Fig. 9) just as characteristic as that seen in tuberculosis.

*The Phosphatide from Bacillus leprae*

The organisms used (No. 370) for fractionation were obtained from the Hygienic Laboratory. The strain was isolated from a leprous human being in Honolulu by Clegg and Curry in 1909. The phosphatide (13) represents 2.2 per cent by weight of these organisms. This fraction has been tested in only four rabbits, two of which received doses smaller than usual on account of the scarcity of the available material. Later two other animals received doses of this phosphatide identical with those from the other mycobacteria.

Rabbits R 1949 and R 1950 (Table IV) received ten doses at 24 hour intervals. Each was killed for autopsy on the day following the last dose. One (R 1950, Table IV) showed a massive reaction of tubercle-like structures made up chiefly of epithelioid cells of the first stage, with smaller numbers of the second, and a few third stage cells (Fig. 10). Many giant cells of complex type were seen. Some were very large with a mass of nuclei usually forming a cap at one side of the cell, and they were possibly formed by the fusion of Langhans giant cells. Numerous plasma cells, small numbers of lymphocytes, and a minimal amount of caseation also characterized the reaction.

The second animal (R 1949, Table IV) showed a cellular reaction much less extensive and of mixed type. Epithelioid cells of the first and second stages were present but did not dominate the picture. There were many monocytes, a great deal of undifferentiated connective tissue, lymphocytes, plasma cells, polymorphonuclear leucocytes, and clasmotocytes.

Rabbit R 2414 (Table IV) received a single dose of 1 gm. of the phosphatide from *B. leprae* intraperitoneally. Supravital studies and fixed tissues revealed no cellular reaction. Rabbit R 2415 (Table IV), however, received ten daily doses of 80 mg. of the same material and showed at autopsy a massive reaction closely resembling that of Rabbit R 1950. The cause of this variation in the reaction to the phosphatide from *B. leprae* has not been determined.

*Reactions to Tuberculo-Phosphatide in Guinea Pigs*

As it has been indicated in the discussion of the myelin-like figures observed in the connective tissue cells after one or two injections of tuberculo-phosphatide, the reaction produced by this substance in guinea pigs was similar to that which occurred in rabbits. The principal difference was that the cells of the guinea pig required more time for the intracellular dispersion of the material into the smallest vacuoles. There was also a moderate stimulation of indifferent connective tissue cells after injections of the phospholipin in guinea pigs.

## LIPOID FRACTIONS FROM ACID-FAST BACILLI

Thirteen animals received the material either intraperitoneally or intrapleurally. Autopsies were performed from 1 to 21 days after

TABLE IV  
*Protocols of Rabbits Which Received Phosphatides from Timothy Grass and Leprosy Bacilli Intraperitoneally*

Animal No.	Source of phosphatide	No. of injections	Amount of each injection	Interval between last injection and autopsy	Observations
R 1427	Timothy grass bacillus	1	mg. 80	days 2	Moderate increase in size of milk spots of omentum. Several small nodules (monocytes and fibrin) free in peritoneal cavity. Milk spots showed many first stage epithelioid cells, monocytes, and primitive cells. There were many polymorphonuclears and clasmatocytes in interspaces and in peritoneal fluid. There was some caseation. Moderate numbers of early epithelioid cells in retrosternal nodes
R 1426	" "	10	80	4	Extensive thickening of omentum. Numerous yellow nodules of epithelioid cells and caseous material in omentum and free in peritoneal cavity. Omental reaction both nodular and diffuse. Many first and second, but few third stage epithelioid cells, a few giant cells, numerous plasma cells, and a few lymphocytes. Extensive reaction in retrosternal nodes
R 1949	Leprosy bacillus	10	80	1	Omentum only slightly stimulated. There were monocytes and epithelioid cells of the first and second stage but polymorphonuclears and clasmatocytes were more numerous. Many plasma cells and undifferentiated connective tissue cells
R 1950	" "	10	80	1	Omentum massively thickened. Marked reaction in retrosternal nodes. Many tubercle-like clumps of first and second stage epithelioid cells, rosette and complex giant cells, many plasma cells, and lymphocytes in omentum. Small amount caseation. A very mixed reaction as regards types of epithelioid cells
R 2414	" "	1	1000	15	Sections of omentum, the retrosternal and tracheal lymph nodes showed moderate numbers of polymorphonuclears and clasmatocytes. No other abnormal findings
R 2415	" "	10	80	3	Massive reaction. In all respects like that in R 1950

injection of the phosphatide. None of the animals showed complete dispersion of the lipoid into the finest vacuoles during this time. Protocols of the guinea pigs which received phosphatide are in Table V.

TABLE V

*Protocols of Guinea Pigs Which Received Phosphatide from Human Tubercle Bacilli*

Animal No.	Route of injection	No. of injections	Amount of each injection	Interval between last injection and autopsy	Observations
			mg.	days	
R 691	Intraperitoneal	1	80	1	Moderate increase in size of milk spots of omentum. In the supravital preparations and fixed tissues the predominating cells were leucocytes, free and in clasmatocytes. There were numerous cells in the omentum with large vacuoles of irregular size and shape. Numerous monocytes. Considerable necrosis
R 692	"	2	80	1	Abdominal viscera covered with a thick white exudate consisting of leucocytes, clasmatocytes, and monocytes. No macroscopic stimulation of omentum. Microscopically there were many leucocytes and clasmatocytes in the interspaces; in the milk spots, many primitive cells, monocytes, and first stage epithelioid cells. Considerable necrosis. Cultures of exudate negative
R 693	"	3	80	1	Moderate amount exudate as in R 692. Omentum markedly thickened. An occasional tubercle-like structure and extensive diffuse reaction. Fewer leucocytes and clasmatocytes than R 692. Numerous monocytes, primitive cells, and first stage epithelioid cells. Considerable amount of necrosis and some typical caseation. Cultures of exudate negative
R 690	"	7	80	1	Many white nodules free in abdominal cavity or adherent to viscera. Very marked stimulation of omentum. Predominating cell was the first stage epithelioid cell with very large vacuoles (Fig. 13), both in omentum and peritoneal fluid. A few epithelioid cells of second stage were present. Moderate amount of caseation. Numerous tubercle-like structures. Moderate increase in undifferentiated connective tissue. Culture of exudate negative
R 689	"	12	80	1	Omentum extremely thickened and adherent to liver, spleen, parietal peritoneum, and diaphragm. Numerous monocytes. Predominating cell was the first stage epithelioid cell with myelin-like figures, although a few second and an occasional third stage epithelioid cells were present. Many tubercle-like structures. Moderate amount caseation. Moderate increase in undifferentiated connective tissue. Cultures of peritoneal exudate negative
R 1953	Intraperitoneal	1	500	Died 6 days	Postmortem changes too extensive for cellular studies
R 1956	"	1	500	" "	" "

## LIPOID FRACTIONS FROM ACID-FAST BACILLI

TABLE V—*Concluded*

Animal No.	Route of injection	No. of injections	Amount of each injection	Interval between last injection and autopsy	Observations
R 1955	Intraperitoneal	1	mg. 500	days 11	Bowel adherent to abdominal wall over area 2 cm. in diameter. Moderate amount white exudate. Omentum markedly thickened. Extensive reaction in retrosternal lymph nodes. Omentum and nodes showed all stages of epithelioid cells, second stage predominating. An occasional giant cell. A few lymphocytes. A little caseation. Many tubercle-like structures. Moderate increase in undifferentiated connective tissue
R 1954	"	1	500	14	Small intestines massively adherent to parietal peritoneum. Peritoneal fluid clear. Omentum massively thickened. Many tubercle-like structures. Massive reaction in retrosternal nodes. Predominating cells were the second stage epithelioid cells with coarse vacuoles of uniform size. Numerous third stage and a few first stage epithelioid cells. A few rosette giant cells and lymphocytes. Numerous plasma cells. Small amount of caseation. Moderate increase in undifferentiated connective tissue
R 1925	"	1	20	20	Each animal showed slight thickening of the omentum. There were several small tubercle-like masses of epithelioid cells and a few giant cells in each omentum. Numerous monocytes, stimulated monocytes, and fewer epithelioid cells in the peritoneal fluid. The majority of the epithelioid cells in the omentum were of the third stage. In each animal there was enlargement of the retrosternal nodes, sections of which showed rather extensive involvement with epithelioid cells. Each showed a slight increase in undifferentiated connective tissue in the omentum. Reaction more extensive in retrosternal nodes than in the omentum. R 1927 showed a little more reaction than the others, and a little caseation
R 1923	"	1	20	20	
R 1924	"	1	20	21	
R 1927	"	1	20	21	
R 2097	Intrapleural	1	20	18	Numerous typical second and third stage epithelioid cells in the pleural fluid. On the pleura was an exudate of the same cells. The retrosternal and tracheal nodes, especially the former, show massive involvement with second and third stage epithelioid cells. Moderate numbers of lymphocytes and a few plasma cells about the epithelioid cells on the pleura
R 2098	"	1	20	18	Observations same as in R 2097

Guinea Pigs R 1925, R 1923, R 1924, and R 1927 (Table V) each received 20 mg. of the phosphatide intraperitoneally and were killed about 3 weeks later. In each instance the reaction was more extensive in the retrosternal nodes (Fig. 11)

than in the omentum, although unequivocal in the latter situation (Fig. 12). On numerous other occasions, animals which have received a single injection have shown the most extensive reaction in the lymph nodes which drain the peritoneal cavity. Quantitatively the omental response to two injections or more has been greater in proportion than that to a single injection. This is in accordance with the observations of Menkin (26). He has observed rapid drainage of foreign substances through the lymphatics from a normal area; but from an area which is the site of an inflammatory reaction, drainage through the lymphatics is inhibited or suppressed.

*Comparison of Cellular Reaction to Phosphatide with That to Killed Tubercle Bacilli and Defatted Bacilli*

Vigorous cellular reactions are induced by parenteral injection of all the fractions of tubercle bacilli thus far tested. It is only the lipoids, however, and particularly the phosphatides, which produce reactions characterized principally by the formation of tubercle-like masses of epithelioid cells. It seemed necessary, therefore, to determine any points of difference or similarity between reactions of the cells to tuberculo-phosphatide and those to killed tubercle bacilli and tubercle bacilli from which a portion of the fat had been removed. The latter represent the bacillary residue after prolonged treatment of the original organisms with alcohol, ether, and chloroform.

Rabbit R 2192 received 100 mg. of heat-killed tubercle bacilli suspended in 2 cc. of distilled water intraperitoneally and was killed 11 days later. Rabbit R 2193 received 100 mg. of defatted tubercle bacilli in 3 cc. of distilled water intraperitoneally and was killed 12 days later. Rabbit R 2255 received 6.5 mg. of phosphatide (the amount derived from 100 mg. of human tubercle bacilli) in 1 cc. of distilled water intraperitoneally, and was killed 10 days later. Striking differences were seen in the qualitative reactions to these three foreign substances. Each produced characteristic tubercle-like structures and scattered epithelioid cells. The reactions produced by the defatted and heat-killed tubercle bacilli were much more complex. The tissues of R 2192 and R 2193 showed many phagocytic clasmatoocytes, leucocytes, and fibroblasts, all of these being more numerous in R 2192, which received the heat-killed bacilli. It is as if the reaction to the phosphatide had been produced by a relatively simple substance, while that to the defatted bacilli and heat-killed bacilli had been induced by more complex substances which have also the capacity to stimulate proliferation of other types of cells than epithelioid cells. This is, indeed, not surprising since both the heat-killed and partially defatted bacilli contain proteins and polysaccharides which cause marked changes in the connective tissues, and which are not contained in the tuberculo-phosphatide.

## LIPOID FRACTIONS FROM ACID-FAST BACILLI

The significance of the element of time with regard to the complexity of cellular reactions to heat-killed and partially defatted tubercle bacilli has been demonstrated in other animals receiving these substances. Guinea Pigs R 518, R 519, and R 520 each received a single intraperitoneal injection of 1 mg. of heat-killed human tubercle bacilli. Guinea Pig R 518 was killed with ether 17 days after the injection. At autopsy the omentum was moderately thickened. There was a moderately extensive reaction limited to the omentum. There were many epithelioid cells, both in clumps resembling tubercles, and scattered diffusely. Many lymphocytes were also present. There were numerous phagocytic clasmatocytes containing debris, but this aspect of the pathological picture was much less pronounced than in Rabbit R 2192. There was moderate proliferation of fibroblasts. This reaction more closely resembled that seen in R 2255 than did the reaction observed in R 2192.

Guinea Pig R 519 was killed 22 days after introduction of 1 mg. of heat-killed human tubercle bacilli. Again the entire reaction was confined to the omentum. There were no epithelioid cells scattered diffusely but numerous tubercle-like clumps of them could be seen. The clasmatocytic response noted in R 518 had disappeared and the reaction closely resembled that seen in animals receiving the phospholipin.

Guinea Pig R 520 was killed 30 days after the introduction of 1 mg. of heat-killed tubercle bacilli. The cellular reaction was confined to the omentum and consisted of many tubercle-like clumps of epithelioid cells, an occasional giant cell, and many lymphocytes. The reaction resembled that seen in R 2255 which received tuberculo-phosphatide and was killed 10 days later.

Rabbit R 1772 received one injection of 0.1 mg. and six injections of 0.2 mg. of defatted bovine tubercle bacilli suspended in 1 cc. of normal saline subcutaneously in the right groin at 3 day intervals. The animal died 48 hours after the last injection. At autopsy a non-tuberculous pneumonia and empyema were found. No acid-fast organisms were found in the lung and in the pus in the pleural cavity. In the inguinal region at the site of the injections there was an abscess of moderate size, containing a few intact acid-fast rods and fragments of them. In sections this abscess had the appearance of a mass of epithelioid cells, the central portion of which had proceeded through caseation to liquefaction.

Rabbit R 1777 received intravenously one injection of 0.1 mg. and six injections of 0.2 mg. of defatted bovine tubercle bacilli suspended in 1 cc. of normal saline at 3 day intervals. The animal was killed 16 days after the last injection. There were numerous small tubercles in the lungs, made up for the most part of rosette giant cells, with smaller numbers of epithelioid cells. A few of the tubercle-like structures had caseous centers. Many lymphocytes were arranged about the periphery of each mass. No appreciable number of fibroblasts or clasmatocytes were present. The only other lesions were in the liver and consisted wholly of isolated rosette giant cells.

It can then be seen that the tissue changes occurring 10 or 12 days after injection of heat-killed or defatted organisms differ from those

occurring at a similar time after the injection of tuberculo-phosphatide in that the former are more complex. However, at a later time the reaction to heat-killed and defatted bacilli becomes simplified by the disappearance of granulocytes and clasmatocytes, so that the tubercular tissue so produced appears much like that induced by tuberculo-phosphatide.

*Antigenic Power of Phosphatides from Mycobacteria*

Pinner (27) first studied the antigenic nature of products from tubercle bacilli. Doan (28) and Doan and Moore (29) then found that the sera of some tuberculous animals and human beings contained precipitins for homologous tuberculo-phosphatide. Boissevain (30) has recently stated that a lipid isolated from human tubercle bacilli by ether extraction produced cutaneous hypersensitivity to tuberculin. He stated, however, that this lipid contained acid-fast bacilli. The phosphatides isolated by Anderson contain no demonstrable acid-fast organisms. However, it was deemed advisable to investigate whether the latter lipid could produce cutaneous hypersensitiveness.

Accordingly two tuberculous animals (R 2229 and R 2230, Table VI) were given 2 mg. each of tuberculo-phosphatide intracutaneously in 0.1 cc. distilled water and were observed for evidence of reaction. Simultaneously skin tests with tuberculo-protein (Ma-100, 0.1 mg. in 0.1 cc. distilled water) were made on the same animals. In addition, normal animals which had received tuberculo-phosphatide (Anderson A-3) were, after an appropriate interval, tested for cutaneous hypersensitivity to homologous tuberculo-protein (MA-100, 0.1 mg. in 0.1 cc. distilled water).

The results of the tests are in Table VI. It can be seen that the phosphatide given intracutaneously produces a nodule which persists for some time, and that this reaction is in no way comparable to hypersensitivity to tuberculin. Moreover, animals which had received phosphatide parenterally did not exhibit cutaneous hypersensitiveness to tuberculo-protein. Boissevain has attributed tuberculin hypersensitiveness and the formation of tubercular tissue to water-insoluble proteins from the tubercle bacillus.<sup>2</sup> Although the water-insoluble protein isolated by Johnson and Coghill (23, 24) had been tested previously in this laboratory (10), additional tests have recently been made.

<sup>2</sup> At the 28th Annual Meeting of the National Tuberculosis Association, in Colorado Springs, June, 1932.



## LIPOID FRACTIONS FROM ACID-FAST BACILLI

The water-insoluble protein, 10 mg. suspended in distilled water, was given intraperitoneally to each of two normal rabbits daily for 10 days. At autopsy on the 11th day there was no tubercular tissue to be found. The protein had induced a cellular reaction characterized by leucocytes, phagocytic clasmatocytes, and smaller numbers of lymphocytes and plasma cells. A water-insoluble aluminum precipitate of a dye-protein compound of high antigenic power and deep color (31), obtained through the courtesy of Dr. Michael Heidelberger, was injected intraperitoneally into a rabbit in 10 mg. doses daily for 4 days. The cellular reaction

TABLE VI  
*Results of Intracutaneous Tests for Hypersensitiveness*

Animal No.	Inoculated with 0.1 mg. human tubercle bacilli	Amount of phosphatide injected and route of injection	Interval between injection and skin tests		Cutaneous reaction to tuberculo-protein		Time required to subside		Cutaneous reaction to tuberculo-phosphatide	Time required to subside		Remarks
			days	tests	++	+	days	tests		days	tests	
R 2229	Nov. 12, 1931		36		++		3		+++	30+		Neither nodule became necrotic. The regression of the phosphatide nodule was delayed and very gradual in both animals
R 2230	" "		36		++		3		+++	30+		
R 1923		20 intraperitoneal	17	0								
R 1924		20 "	17	0								
R 1925		20 "	17	0								Tuberculo-protein MA-100 and old tuberculin used. Both tests negative in each instance
R 1927		20 "	17	0								
R 2355		40 subcutaneous	22	0								
R 2356		50 "	22	0								
R 2357		50 "	22	0								Tests made with tuberculo-protein MA-100. All entirely negative
R 2358		50 "	22	0								

which ensued was studied. The animal exhibited a response similar in all cytologic aspects to the two animals receiving the water-insoluble tuberculo-protein. The living cells were studied without neutral red or other accessory staining and the ingested protein was recognized within the cells by the dye which had been introduced into the protein molecule.

From our studies, it appears that water-insoluble protein is not associated with the formation of tubercular tissue.

## DISCUSSION

It now seems certain that the changes produced in the tissues by the Anderson phosphatides are initiated by substances which are relatively

pure in the biological sense, and not by bacteria which have withstood the processes of extraction and filtration. No such bacteria or fragments are demonstrable in stained preparations of these phosphatides. In addition, it is now known that the purified phosphatide is predominantly crystalline. It is well known that dead, as well as living tubercle bacilli cause hypersensitiveness to tuberculin in guinea pigs. Cutaneous sensitization has not been elicited with tuberculin after introduction of the Anderson phosphatide. Probably more significant still is the fact that the reactions of the tissues of animals to the phosphatides are characterized chiefly by one type of cell, the epithelioid. In the light of the observation of Sabin, Doan, and Forkner (9), that lecithin (from brain) produces a similar qualitative reaction, strength is given to the hypothesis that the epithelioid cell is a connective tissue cell which has phagocytized a lipoidal substance. The appearance of the cell suggests an emulsification of the lipid.

The bacterial residue which remains after extracting human tubercle bacilli for 4 weeks with a mixture of alcohol, ether, and water, and then with chloroform, retains to some degree the property of acid-fastness. The partially defatted organisms produced tubercular tissue when injected into animals. After the phosphatide is precipitated from the ether solution with acetone, a considerable quantity of lipid remains in solution. This partition, designated the acetone-soluble fat, has also been observed to produce epithelioid cells when injected into animals. The phosphatide, which constitutes 6.54 per cent of the human tubercle bacillus, is but one of three substances obtainable from these organisms having the capacity to produce epithelioid cells. It cannot, therefore, be expected that the phosphatide will reproduce quantitatively the reaction caused by the same weight of the tubercle bacilli from which it is derived. Nevertheless, the reaction to the phosphatide is remarkably pure, tends principally toward one type of cell, and is qualitatively specific.

The fact that a thin film of phosphatide which has been mixed with water assumed forms which, microscopically, closely resemble degenerating myelin has made possible a determination of the fate of this material after injection. The lipid is phagocytized by monocytes, or, if the stimulus be great enough, by the primitive cells which give rise to monocytes. After the lipid has been phagocytized it is subjected to a process of intracellular dispersion into finer and finer droplets. After

reaching the stage of the finest droplets, no further detectable change takes place. While this process of phagocytosis and intracellular dispersion is going on, the cells exhibit evidence of proliferation and maturation, it being plain that all the functions of the cell are simultaneously set into action.

In the further analysis of the lipoids from mycobacteria, Dr. Anderson (13) has obtained by hydrolysis of the phosphatide certain fatty acids of high molecular weight. When injected into animals these fatty acids cause the formation of tubercular tissue (9). However, the epithelioid cells produced by the fatty acids pass directly to the fine vacuole stage. It therefore seems probable that the intracellular dispersion of tuberculo-phosphatide may represent a process of intracellular hydrolysis. In the one instance the process takes place *in vitro*, in the other *in vivo*.

The phospholipins from avian and bovine tubercle bacilli, timothy grass and leprosy bacilli produce reactions simulating those which occur after injecting the same fraction from human tubercle bacilli. The principal difference is that the process of intracellular dispersion of the lipid takes place at a slower and more irregular rate. If this phenomenon of intracellular degradation represents hydrolysis, then the irregularity with which it occurs in the instances now under discussion may be but the expression of differences in chemical composition of the injected lipid, by virtue of which it is subdivided with greater difficulty by the cells. Moreover, the fact that degradation of the phosphatide from human tubercle bacilli takes place more slowly in the cells of guinea pigs than in rabbits may indicate a greater abundance in the latter of some chemical factor necessary for the reaction.

Anderson (13) has shown that the nitrogen content of the bacterial phosphatides does not exceed 1.0 per cent (bovine) and that the value for the phosphatide from human tubercle bacilli is 0.36 per cent. He has also found the greater part of this nitrogen can be easily removed as ammonia. Since it requires a considerable amount, relatively speaking, of tuberculo-protein and a somewhat protracted series of injections to produce cutaneous hypersensitiveness to tuberculin, it is not surprising that the Anderson phosphatides do not cause cutaneous hypersensitiveness.

## SUMMARY

1. A comparative study has been made of the cellular reactions induced by phosphatides from five strains of acid-fast bacilli. Each of these reactions is characterized principally by epithelioid cells and giant cells.

2. The phosphatides are first phagocytized by young connective tissue cells or monocytes. The lipid is then dispersed into fine particles with the formation of classical epithelioid cells.

3. A comparison has been made of the reactions induced by heat-killed and defatted tubercle bacilli with those induced by tuberculo-phosphatide.

4. Further studies have been made to determine whether or not the phosphatide causes sensitization to tuberculin. It does not do so.

5. The life cycle of the epithelioid cell has been observed in all its stages.

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#### EXPLANATION OF PLATES

##### PLATE 44

FIG. 1. A preparation of living cells from the peritoneal fluid of Rabbit R 1802 which had received three intraperitoneal injections of 80 mg. of the phosphatide from human tubercle bacilli. Note large vacuoles of irregular size and shape in the first stage epithelioid cells. Beneath this cell is a granulocyte. The cells at the right are monocytes. Note the delicate surface films. Supravital neutral red and Janus green.  $\times 1200$ .

FIG. 2. Section of the omentum of Rabbit R 821 after three intraperitoneal injections of 80 mg. of phosphatide from H-37. The two cells with highly vacuolated cytoplasm are first stage epithelioid cells or the same type shown in the living state in Fig. 1. The two smaller cells are monocytes. Hematoxylin and eosin.  $\times 1200$ .

FIG. 3. A film of living omentum from Rabbit R 2186 showing an epithelioid cell of the second stage. Note that the vacuoles are of uniform size and shape, but smaller than in the cell in Fig. 1. Supravital neutral red and Janus green.  $\times 1300$ .

FIG. 4. Section of the omentum from Rabbit R 2186, showing epithelioid cells of the second stage from the same animal as that of Fig. 3. Note foamy appearance of cytoplasm. Hematoxylin and eosin.  $\times 1200$ .

FIG. 5. Section of the omentum of Rabbit R 377, 90 days after thirteen injections of 80 mg. of phosphatide from H-37. The cell in the center is a degenerating epithelioid cell. Note indefinite cytoplasmic outline, pale vacuolated cytoplasm, and disintegrating nucleus. The other cells are third stage epithelioid cells. Hematoxylin and eosin.  $\times 1200$ .

FIG. 6. Section of the omentum of Rabbit R 2187 after ten intraperitoneal injections of 80 mg. of phosphatide from H-37. Note homogeneous appearance of cytoplasm of the third stage epithelioid cells. Hematoxylin and eosin.  $\times 1200$ .

## PLATE 45

FIG. 7. Section of omentum of Rabbit R 688 after twelve injections of 80 mg. of the phosphatide from avian tubercle bacilli. The epithelioid cells are of the second and third stages. A few lymphocytes are in the lower portion of the photograph. Hematoxylin and eosin.  $\times 1000$ .

FIG. 8. A section of the omentum of Rabbit R 1015 after eleven intraperitoneal injections of 80 mg. of phosphatide from bovine tubercle bacilli. A large rosette giant cell is in the center, and about it epithelioid cells of the second and third stages. Hematoxylin and eosin.  $\times 1000$ .

FIG. 9. A section from the wall of the cecum of Rabbit R 1426 after ten intraperitoneal injections of 80 mg. of phosphatide from timothy grass bacilli. It shows a nodule of tubercular tissue with caseous center and intact epithelioid cells about the periphery. Hematoxylin and eosin.  $\times 240$ .

FIG. 10. A section from the omentum of Rabbit R 1950 after ten intraperitoneal injections of 80 mg. of phosphatide from leprosy bacilli. Note that all of the epithelioid cells are highly vacuolated. Hematoxylin and eosin.  $\times 1300$ .

## PLATE 46

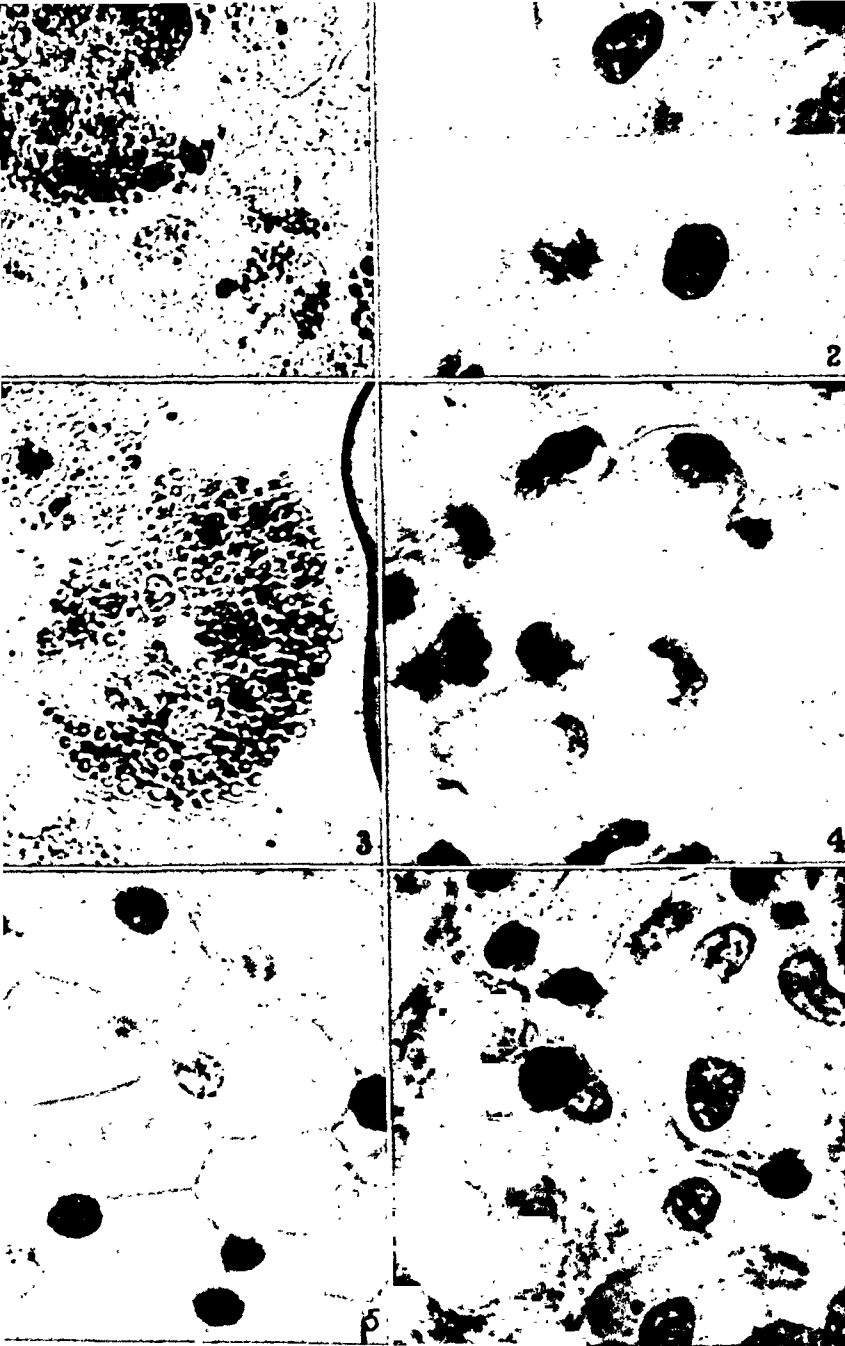
FIG. 11. A section from the retrosternal lymph node of Guinea Pig R 1927, 3 weeks after a single intraperitoneal injection of 20 mg. of phosphatide from human tubercle bacilli. Note epithelioid cells surrounding a lymphoid follicle. Hematoxylin and eosin.  $\times 250$ .

FIG. 12. A section from the omentum of Guinea Pig R 1927 (same animal as of Fig. 11), showing epithelioid cells and giant cells beneath the serosa. Hematoxylin and eosin.  $\times 250$ .

FIG. 13. A living preparation of peritoneal exudate from Guinea Pig R 690 after seven intraperitoneal injections of 80 mg. of phosphatide from human tubercle bacilli. Note myelin-like figures in the two large cells, those in the upper one being smaller than those in the lower cell. Supravital neutral red and Janus green.  $\times 1050$ .

FIG. 14. A section of the omentum from the same guinea pig as that in Fig. 13. Note highly vacuolated appearance of cytoplasm in the epithelioid cells, and compare with the appearance of the same cells in the living preparation (Fig. 13). Hematoxylin and eosin.  $\times 1000$ .



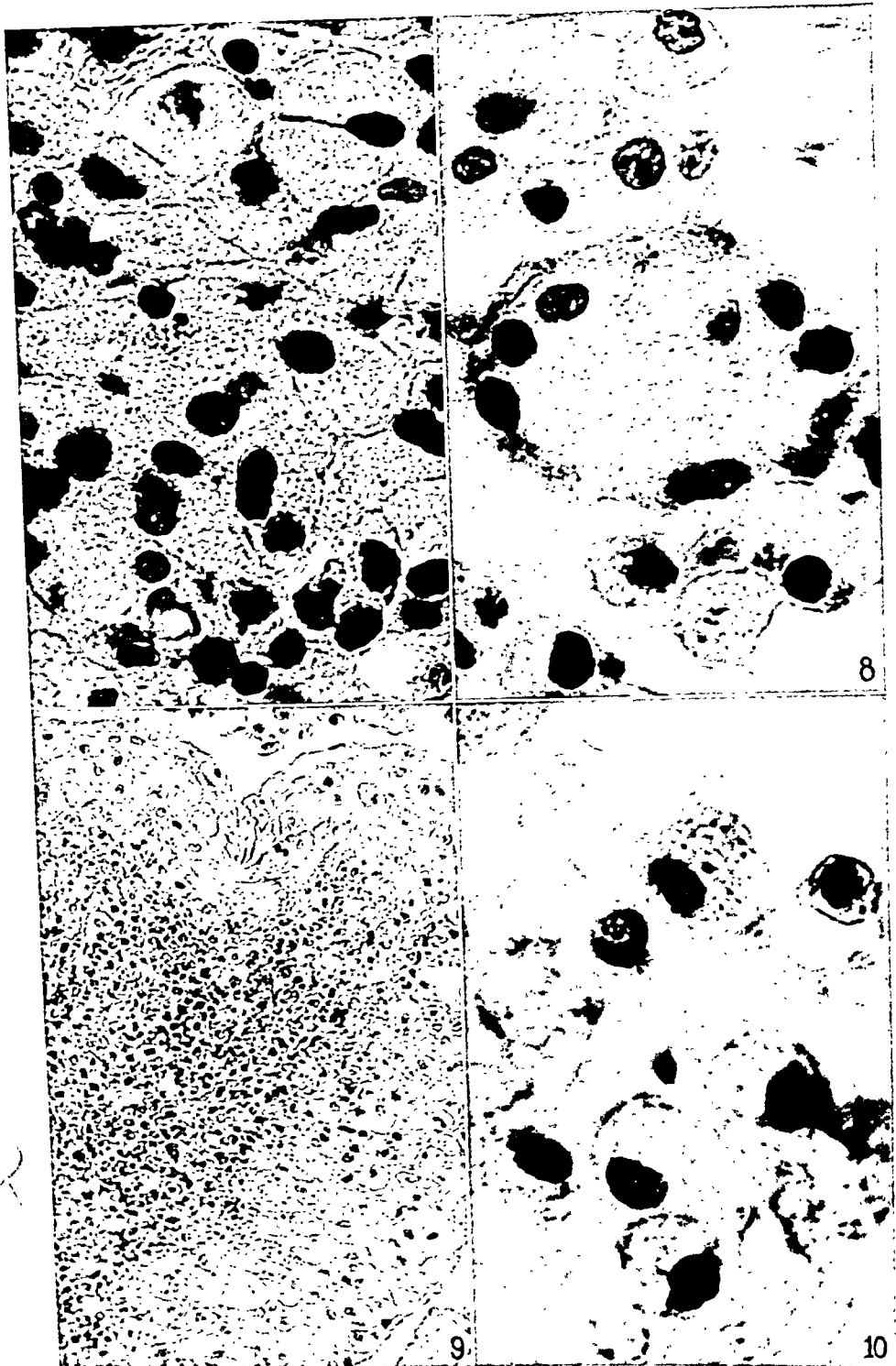


Prepared by Louis Schmitt

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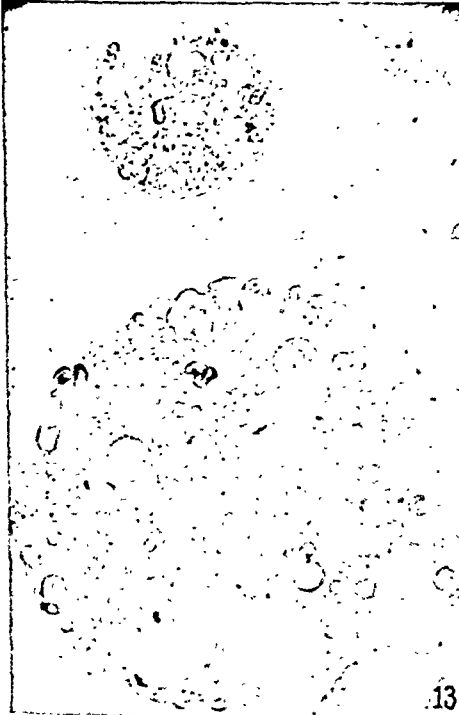
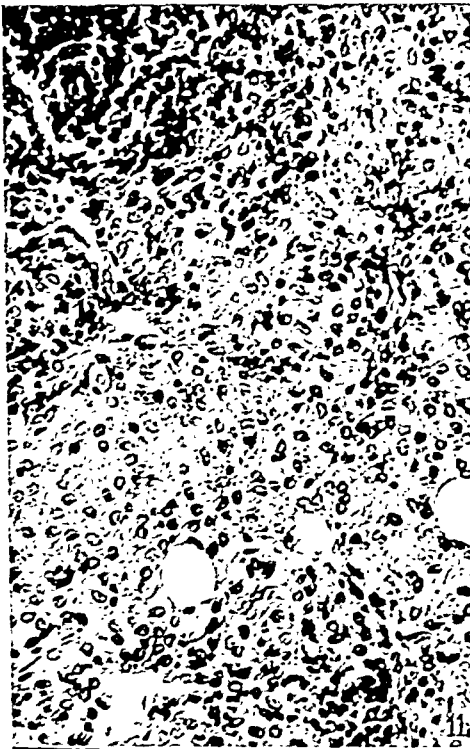




Photographed by Louis Schmidt

(Smithburn and Salinas: Lipid fractions from acid-fast bacilli)







## SOME PHYSIOLOGICAL CHARACTERISTICS OF EPITHELIAL TUMORS OF THE MOUSE

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PLATES 47 TO 49

(Received for publication, June 1, 1932)

In order that an accurate comparison might be made of the properties of normal and malignant tissues *in vitro*, it was necessary to study tissues of identical origin. For such a purpose, the spontaneous epithelial tumors of the mouse, originating from the mammary gland, are most suitable. However, as a technique for the successful cultivation of normal mammary epithelium has not yet been perfected, such an investigation had to be limited to the characteristics of various types of tumors.<sup>1</sup> According to Apolant,<sup>2</sup> Murray,<sup>3</sup> and others, mouse tumors are histologically of either benign or malignant nature. The relations between their microscopical structure and their physiological properties as manifested *in vitro* are still unknown. But in a previous article from this laboratory,<sup>1</sup> certain differences in the behavior *in vitro* of epitheliums from diverse tumors were mentioned. The purpose of the studies described in the present paper was to ascertain the significance of these differences.

*Histological*

A number of tumors were studied histologically, and cultivated *in vitro* by the technique previously reported.<sup>1</sup> The microscopical structure of spontaneous mammary gland tumors of the mouse was thoroughly described in 1906 by Apolant,<sup>2</sup> whose findings and classification have in general been confirmed and adopted by later investigators,

<sup>1</sup> Santesson, L., *J. Exp. Med.*, 1932, 55, 281.

<sup>2</sup> Apolant, H., *Arch. k. Inst. exp. Therap. Frankf. a.M.*, 1906, 1, 7.

<sup>3</sup> Murray, J. A., *3rd Scient. Rep. Int. Imp. Cancer Research Fund*, London, 1908, 69.

notably Murray,<sup>3</sup> Gierke,<sup>4</sup> and Haaland.<sup>5</sup> According to Apolant,<sup>2</sup> these tumors form one large group, whose members have gradually developed from a common origin by a differentiation which has taken place in various directions. Although the stages of this differentiation are far from being sharply defined, it is still possible, according to Apolant, to distinguish two main types, adenomas and carcinomas.

The tumors discussed in the present article occurred among a large group of mice, about 14,000 in number, composed of four strains, Rockefeller Institute, Swiss, Lathrop, and Dilute Brown.<sup>6</sup> Macroscopically, the tumors were mostly well defined, encapsulated, and solid. Microscopical examination of over 1,300 mammary gland tumors proved that the findings of Apolant hold true, and that a histological classification is possible. Nevertheless, the types are not sharply distinct, and such a classification must be considered artificial to some extent. The tumors cultivated *in vitro* have been found to belong to one of the following groups: adenomas, adenocarcinomas, and carcinomas.

*Adenomas.*—These tumors are macroscopically homogeneous (Fig. 1). They contain, as most epithelial mouse tumors do, only a very small amount of stroma, and consist of round or elongated alveolar structures, closely corresponding in appearance to the acini of the normal mammary gland. Like these, they are composed of small cells, uniform in appearance, and organized in single layers. Mitoses are seldom observed. The acini lie compactly together, and are mostly without any recognizable septa in the central parts of the tumors. Some tumors contain a more or less abundant round cell infiltration. In the central parts of the tumors, there is a tendency to secondary alterations such as cyst formation, edema, necrosis, or hemorrhage, the adenomatous structure being still generally maintained. Carcinomatous degeneration may also occur. To judge from their histological picture, these tumors are of a highly organized, benign character. They are the first stage in the development of mammary gland carcinomas, as Apolant<sup>2</sup> has clearly shown.

<sup>4</sup> Gierke, E., *3rd Scient. Rep. Inv. Imp. Cancer Research Fund*, London, 1908, 115.

<sup>5</sup> Haaland, M., *4th Scient. Rep. Inv. Imp. Cancer Research Fund*, London, 1911, 1.

<sup>6</sup> The tissues were fixed in 10 per cent formalin, sectioned in paraffin, and stained with hematoxylin and eosin.

*Adenocarcinomas.*—When the adenoma undergoes carcinomatous degeneration, the epithelium proliferates into the lumen of the acini, and fills it. It thus constitutes a solid mass of epithelial tissue (Fig. 2). This atypical growth starts generally inside a typical adenoma. It produces a nodule which is sharply defined and easily recognizable. In the early stages, mitoses are more abundant in these parts. Such solid epithelial nests often arise simultaneously in many places, expand, and coalesce as the process goes on and, in the end, form a typical carcinoma throughout the whole tumor. It is only rarely, however, that this process is complete. Generally, more or less extended carcinomatous regions are seen beside typical adenomatous structures, the whole constituting an adenocarcinoma. This group forms a connective link between the adenomas and the typical carcinomas.

*Spontaneous Carcinomas.*—Tumors of this group are typically carcinomatous (Fig. 2). Adenocarcinomas, as well as carcinomas, show wide variations in their histological appearance. In adenocarcinomas, the amount of atypical proliferation may vary greatly. In carcinomas and in the carcinomatous parts of adenocarcinomas, every gradation was observed, from a regular structure composed of cells of equal size and appearance with only a few mitoses, to a highly malignant tissue with many mitoses and abnormal cells in disorganized formations. A tumor histologically benign in one of its parts may be of a highly malignant nature in another part. In the carcinomatous tumors, degenerative changes are observed to a much higher degree than in the adenomatous, either in some parts or in the whole tumor. The amount of stroma and round cell infiltration also varies widely for every tumor. Thus, the appearance of these tumors is far from being uniform. The groups are not sharply defined, especially as far as adenocarcinomas and carcinomas are concerned.

*Transplantable Carcinomas.*—Tumor 1 is an adenocarcinoma, about 50 per cent of the tissue being adenomatous and the rest carcinomatous. It shows few mitoses and but little tendency to cell abnormalities. Tumor 2 is a typical carcinoma, with many mitoses, cell abnormalities, and disorganization. But there is no necrosis. The Ehrlich carcinoma is a highly malignant tumor. It shows a marked tendency to necrosis, cell irregularities, and disorganization, and is usually composed of a mass of dead tissue with a thin infiltrating edge.



## EXPERIMENTAL

The studies have been confined to fresh cultures of tumors, as no pure strains have been maintained alive for long periods.<sup>1</sup> The tumors were removed from the animal as soon as they were discovered.<sup>7</sup> They were comparatively small, with a minimum degree of degeneration. Tissues which were necrotic, hemorrhagic, or contained cystic formations were cultivated only if homogeneous fragments could be obtained. They were cut into small pieces, as nearly as possible of identical size, about 0.5 to 1.0 mm. long, and washed in Tyrode solution. Fragments for histological examination were taken at the same time, always from parts adjacent to the explants.

The usual method of cultivation in Carrel flasks was employed. The following culture media were used. Solid phase: 1.0 cc. of chicken plasma, diluted 1:8 with Tyrode solution, coagulated with 2 drops of chick embryonic juice, and washed twice with 3.0 cc. of Tyrode solution for 1 hour. Fluid phase: 0.5 cc. of rat serum diluted to 50 per cent with Tyrode solution. Every 2nd or 3d day, the fluid was removed, the clot reinforced, if necessary, with 0.5 cc. of chicken plasma 1:8 plus 2 drops of chick embryonic juice, and washed 1 hour, as described above, after which fresh fluid medium was added. At least twelve pieces of every tumor were explanted, four in each flask. As no accurate knowledge of the proteolytic activity of the cells can be obtained from the mere observation of the liquefaction of the coagulum, a special test had to be developed. Coagulated egg white was cut into slices 35 microns thick in a freezing microtome. The slices were not transparent. When tumor fragments were placed in a culture medium on the surface of these slices, they attacked the protein. Proteolysis was easily recognized by the appearance of a transparent area around the tissue, while the other parts of the slice remained opaque. This test was applied as a routine technique to ascertain the proteolytic activities of the cells. The following procedure was used. At first, 0.25 cc. of diluted plasma was introduced into the flask. The tiny sheets of egg albumin were placed in the medium and carefully spread out. Then, a fragment of tumor was put on each square. Close to each slice, another fragment served as a control. After the plasma was coagulated, the remaining part of the medium was added. Other cultures without egg albumin were always prepared at the same time.

The acid production was studied by cultivating the tumors in a coagulum containing phenol red. This indicator has proved reliable in revealing the changes in the pH which occur in such experiments.<sup>8</sup> In the preparation of the cultures, chicken plasma and rat serum diluted with Tyrode solution containing 0.04 per cent of phenol red were used. There was no inhibiting effect of the dye at such a concentration. The coagulum was washed with Tyrode solution also containing phenol red. Prepared in this way, the medium assumed a bluish red color, corre-

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<sup>7</sup> All operative procedures were carried out under full ether anesthesia.

<sup>8</sup> Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1928, 48, 105.

sponding to a pH of 7.8 to 8.0, as determined by comparison with a set of standard solutions kept in flasks of the same type. The degree of acid production could be judged by determining and comparing the color of various explanted pieces, and also by studying the color change in the surrounding coagulum. It must be remembered, however, that this method only permits the detection of marked differences in acid production. Based as they are on judgment of variations in color, the findings obtained must be accepted with great caution.

### *Physiological Characteristics of the Tumors*

From the 1,300 spontaneous tumors histologically studied, 77 were cultivated *in vitro*, 28 being adenomas, 31 adenocarcinomas, and 18 carcinomas. The characteristics of these tumors were compared with those of 2 transplantable carcinomas and of Ehrlich carcinoma.

*Architecture of the Cultures.*—The first phenomenon was the migration from the tumor fragment of ameboid cells resembling monocytes. These cells usually appeared a few hours after explantation and rapidly spread into the medium. Later, and almost simultaneously with epithelial migration, typical fibroblasts wandered out. The volume and extent of the migration of fibroblasts and ameboid cells varied with each tumor, and corresponded closely to the amount of leucocytic infiltration and of stroma observed in histological sections of the original growth. In no case were amebocytes or fibroblasts more active than could be expected from the microscopical findings. It was obvious that the migrating macrophages and fibroblasts originated from the blood cells and the stroma present in the tumors. Adenocarcinomas and carcinomas generally contained more stroma and blood cells than adenomas. These tumors became surrounded by many ameboid cells and fibroblasts, while adenomatous tissues showed hardly any of these cells. In five cases, adenomas produced epithelial cells exclusively, without any amebocytes or fibroblasts. Each cell type migrated in its own manner. The fibroblasts were less active than the epithelial cells and were always overgrown by them. The ameboid cells degenerated after 3 or 4 days, while the fibroblasts ceased migrating and later on disintegrated.

The first sign of epithelial migration was observed after about 10 to 12 hours. It consisted of a rounding of the edges of the explants. Shortly after, epithelial expansion set in, with membrane-like formations spreading out concentrically from the explants. But obvious

differences were observed in each group of tumors. In the pure adenomas, epithelial migration assumed the appearance of large membranes surrounding the explant (Figs. 4, 8). The cells organized in a thin, even pavement without any tendency to form double layers. The outline of the membrane was sharply defined. Tubulous formations were never observed. Expansion was rapid. The individual cells were healthy and uniform in appearance. They showed no irregularities. They lay close together, without any visible cell borders. No necrotic cells or areas were observed. The cells remained in intimate contact, and never scattered as isolated units. In spite of the rapid growth, very few mitoses were noted. This behavior closely resembled that of the cultures from the normal gland. In fact, no differences between the normal and adenomatous cell types could be detected. This sort of migration went on during the entire period of cultivation.

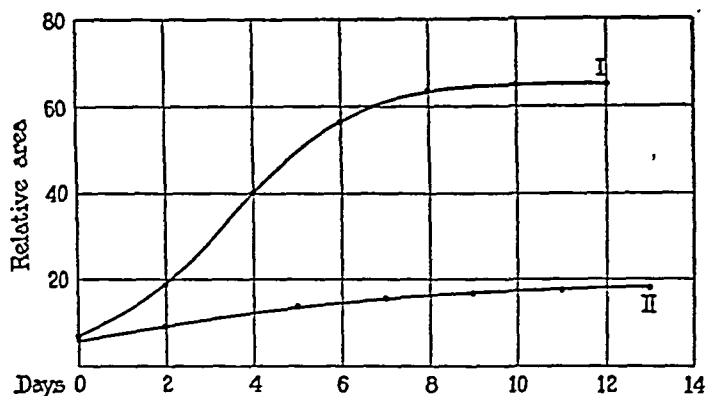
In the group of adenocarcinomas, 23 tumors acted like adenomas, and 8 like carcinomas.

The group of spontaneous carcinomas was composed of 18 tumors. During their growth *in vitro*, 12 of these tumors displayed the following characteristics: Migration was irregular. Processes like buds or broad fingers grew asymmetrically from the edges of the explants, often in double layers. These processes united after some time and formed a membrane. This mode of expansion was so typical that, after 24 hours' cultivation, the nature of the tumor could be diagnosed. Although the cells were organized in regular pavement-like formations, they showed a tendency to differ in size. Giant cells and double nucleated cells were observed. The carcinomatous membranes contained many cells in mitotic division, while in adenomas mitosis was rarely seen.

Transplantable Carcinoma 1 migrated as a membrane in a single layer, slightly liquefying the medium. Carcinoma 2 grew in buds and double layers, and displayed a more irregular architecture and more mitoses than Tumor 2. Ehrlich carcinoma grew very much like the other carcinomas (Figs. 5 and 9). The abnormalities, however, were more marked. The epithelial growth was always irregular and formed membranes consisting of several layers of cells and of bud-like processes. The membranes expanded and presented an uneven, ragged

outer edge. They appeared thicker than those of cultures from spontaneous and transplantable tumors, and were composed of densely crowded and irregularly organized cells. The number of mitoses was much greater and the tendency to cell abnormalities and to necrosis was far more marked than in cultures of spontaneous carcinomas.

*Duration and Rate of Growth.*—When the nutritive medium was composed of Tyrode solution containing 50 per cent rat serum, the onset of epithelial growth varied according to the different types of tumors. Epithelial activity in all spontaneous tumors decreased after 7 or 8 days' cultivation, and ceased almost entirely after about 9



TEXT-FIG. 1. Areas of epithelial migration (I) from 16 adenomas, and (II) from 6 Ehrlich carcinomas, cultivated in 50 per cent rat serum.

days, even if the cultures were provided with fresh fluid medium. Cell migration occurred at the expense of the original fragment, which became gradually thinner as the membrane expanded. This was found to be the case particularly for the adenomas and most of the adenocarcinomas. The carcinomas showed this phenomenon to a lesser extent. They appeared to be a transition type between adenocarcinomas and Ehrlich carcinoma, where fragments constantly increased in size. In the case of spontaneous tumors, the explants did not produce new tissue. They merely spread. When adenomas were cultivated, epithelial migration started after some 10 or 12 hours, and the rate of growth was very rapid. The rate was measured in the usual way, and is shown in Text-fig. 1. In spontaneous and Ehrlich car-

cinomas, epithelial migration invariably began after 20 to 22 hours, that is, much later than in the case of the adenomas, and the rate was slower, as is shown in Text-fig. 1.

*Liquefaction of Fibrin and Digestion of Egg Albumin.*—Earlier experiments<sup>1</sup> showed that a coagulum from mouse, rat, or chicken plasma was readily attacked by epithelial cells migrating from spontaneous tumors. The mode of liquefaction of the medium varied according to differences in the epithelial types. Mouse and rat fibrins were found to be more easily digested than chicken fibrin. In the experiments in which the coagulum was composed of washed chicken plasma, digestion sometimes occurred. At its beginning, it could be detected by a depression in the surface of the clot just above the explants. This depression progressively grew larger and deeper. However, such a manifestation of the proteolytic activity of the tissue did not allow a precise comparison of the various types of epithelial cells. When the digestion of thin slices of egg albumin was used as a test, more accurate results were obtained. Liquefaction of the fibrin and digestion of egg albumin were studied for each type of tumor (Fig. 3).

In adenomas, both proteolytic manifestations were observed to be parallel (Figs. 3 and 10), and were very marked. Fragments from 18 adenomas were cultivated on egg albumin slices, according to the method described above. After 10 to 15 hours, digestion was observed in the form of a transparent area surrounding the explants, while the other parts of the albumin sheet remained opaque. Proteolysis corresponded to epithelial migration. When the growth was rapid, the proteolysis was always marked. When the migration was slow or non-existent, as happens in embryonic extract or Tyrode solution, little or no proteolysis occurred. But if after 48 hours, the medium was replaced by rat serum, which caused rapid epithelial extension, digestion took place. When tumor fragments were explanted near an albumin sheet, the wandering epithelial membrane reached its border after 1 or 2 days, and produced a transparent zone in the form of a half-moon, which increased in size as the membrane spread.

Several adenocarcinomas were found to differ markedly in their proteolytic activities. The egg albumin test was used in 20 cases. Proteolysis first appeared after from 10 to 15 hours up to 4 days, and was equal to or less marked than that produced by the adenomas.

From the point of view of their proteolytic activity, adenocarcinomas closely resembled adenomas.

Similar experiments were performed on 11 carcinomas. In 4 cases, there was no observable digestion. In 4 other cases, digestion appeared after 5 to 6 days, remaining very slight. The liquefaction of the medium was correspondingly slight and did not take place at all in a few tumors. In the last 3 tumors, proteolysis occurred earlier and was more marked, although still slight.

Twelve tumors of the Ehrlich carcinoma strain were cultivated on albumin sheets (Figs. 3 and 11). Very slight liquefaction of the coagulum took place with hardly any digestion of egg albumin, although the epithelium grew extensively.

*Acid Production.*—The production of acid was studied by cultivating the explants in media containing phenol red. As in the case of proteolysis, the amount of acid was closely related to the activity of the epithelial cells. After 15 to 30 minutes, the fragments of adenomas assumed a bright yellow color, corresponding to a pH of about 6.0 to 6.4. Simultaneously with epithelial migration, the yellow color spread in the medium surrounding the explants. This yellow area was much larger than that of the tissue, and contrasted markedly with the bluish red color of the medium. When embryonic chick juice or pure Tyrode solution was used as fluid medium, the fragments showed a red color, corresponding approximately to a pH of about 7.4 to 7.8. But if the fluid medium was removed and replaced by rat serum, the explants readily changed to a bright yellow. Acid production by adenomas was always high. In adenocarcinomas, it was more variable, but always corresponded to the rate of epithelial expansion. The carcinomas, including Ehrlich carcinoma, also presented a yellow color. They were never more acid than adenomas, and generally much less. The size of the yellow area was always smaller than in the case of adenomas.

*Invasion of Normal Tissues.*—It has been shown in this laboratory<sup>1</sup> that spontaneous mammary gland tumors, unlike Ehrlich epithelium, do not infiltrate and overgrow normal tissues *in vitro*. However, slight differences were observed in the behavior of epithelium of different types. In order to ascertain whether these differences were related to the histological structure, embryonic mouse tissues were added

to explants from various tumors. The results were similar to those described in a previous article.<sup>1</sup> Spontaneous tumors either failed to invade the added tissues, or invaded them very slowly, whereas epithelium from Ehrlich carcinoma readily overgrew normal tissues.

Adenomatous epithelium from 4 tumors maintained its regular outline, either pushing away the embryonic fibroblasts, or becoming embedded amidst them. In such a case, the fibroblasts formed a structure like a capsule around the epithelial colony (Fig. 6). No bud-like formations were observed. Three adenocarcinomas gave similar results, except that one of the explanted pieces produced very thin, elongated tubules. Only 1 carcinoma was studied. In every explant, the epithelium advanced in bud-like formations or tubules into the embryonic tissue and, in one case, grew into it for more than a month. Ehrlich epithelium rapidly invaded the embryonic explant in thicker, bud-like structures (Fig. 7), and infiltrated it entirely in about a month.

#### DISCUSSION

A comparative study has been undertaken of the physiological characteristics *in vitro* of 77 spontaneous epithelial mammary gland tumors of the mouse, 2 transplantable mammary gland tumors, and a strain of Ehrlich's carcinoma. The properties of the epithelial cells have been found to depend on the histological type of the tumors (Table I). Obvious differences were observed in the architecture of the colonies, according to the adenomatous or carcinomatous nature of the explants. The adenomas expanded evenly in a single layer, without any cell irregularities or necrotic parts. There were very few mitoses. The carcinomas grew in a more irregular manner, with a tendency to form double layers, buds, and tubules. They showed some cell irregularities and mitoses. Ehrlich carcinomas contained a great many mitoses and abnormal cells, growing unevenly and in disorganized formations. The rate of epithelial expansion in rat serum was found to be invariably more rapid in the cultures of adenomas than in those of carcinomas, especially of Ehrlich carcinoma. The presence of fibroblasts and of ameboid cells did not modify the rate of growth (Text-fig. 1). It was evident that epithelial migration was less rapid in malignant than in benign tumors. This confirmed the

TABLE I

*Characteristics of Mammary Gland Tumors Cultivated in Flasks*

Medium: diluted chicken plasma clot bathed in rat serum and washed at intervals with Tyrode solution

	Pure adenoma (28)*	Adenocarcinoma (31)*	Transplanted adenocarcinoma (2)	Pure carcinoma (18)*	Transplanted carcinoma (15)*	Ehrlich carcinoma (26)*
Cell morphology.....	Uniform cells of healthy appearance. No necrosis. Very few mitoses	Varying from pure adenoma to pure carcinoma	Like pure adenoma	Cells of healthy appearance. Some abnormal- ities. Mitoses	Like pure carcinoma	Abnormal cells. Necrosis. Many mitoses
Type of epithelial mi- gration.....	One-layer men- brane	" "	" "	Tendency to form buds and double layers	" "	Membranes ex- panding in buds and double layers
Rate of epithelial mi- gration (first 6-7 days).....	++-++	++-++ to ++	++-++	++	++	++
Invasive power.....	None	None or very slight	None	None or very slight	Slight	Marked
Effect on chicken fi- brin.....	++-++	++-++ to +	++-++ to ++	++ to +	+	+ to 0
Effect on egg albumin..	++-++	++-++ to +	++-++ to ++	++ to +	+	+ to 0
Production of acid.....	++-++	++-++ to ++	++-++	++-++ to ++	++	++

\* Indicates number of cultivated tumors. At least 12 explants of each tumor were used.



findings of Fischer and Laser,<sup>9</sup> in the case of epitheliums from Ehrlich carcinoma and from normal chickens. The various types of tumors differed markedly as regards both liquefaction of the coagulum and digestion of egg albumin. While adenomas actively attacked egg albumin and fibrin, the digestion produced by carcinomas appeared later and was less marked. In the case of Ehrlich carcinoma, it was lacking almost entirely. Proteolysis was related to the migrating activity of the epitheliums. The adenomatous membranes, while actively digesting egg albumin, were composed of healthy cells. No degeneration occurred, either in individual cells or in any area. On the other hand, Ehrlich carcinomas always contained disintegrating or necrotic regions. The supposition that liquefaction of the fibrin, produced by many varieties of tissues, is due to ferments set free by degenerating cells does not hold true in the case of these tumors.<sup>10-12</sup> It is a remarkable fact that histologically benign tumors digest egg albumin and fibrin more actively than more malignant tumors do.

Spontaneous tumors,<sup>1</sup> unlike Ehrlich carcinomas, do not invade normal tissues *in vitro*. However, a carcinoma, transplanted a few times *in vivo*, showed a detectable though very slight tendency to infiltrate fragments of embryonic tissues. There was always a sharp difference between the reaction of benign and malignant tumors to normal tissues *in vitro*. The production of acid was invariably more marked for adenomatous than for carcinomatous tissues. Although the method of estimating acid production is very crude, the results were so constant and striking that they cannot be attributed to technical errors. It is certain, at least, that carcinomatous cells do not set free more acid *in vitro* than adenomatous ones. The behavior of the tumors after transplantation does not always correspond to the degree of malignancy indicated by their histological structure. According to Murray,<sup>3</sup> Haaland,<sup>5</sup> and Bashford,<sup>13</sup> highly differentiated mouse

<sup>9</sup> Fischer, A., and Laser, H., *Z. Krebsforsch.*, 1927, 26, 80.

<sup>10</sup> Fischer, A., *Gewebezüchtung*, Munich, Rudolph Müller and Steinicke, 3rd edition, 1930.

<sup>11</sup> Policard, A., *Bull. histol.*, 1925, 2, 101.

<sup>12</sup> Zakrzewski, Z., *Z. Krebsforsch.*, 1929, 30, 106.

<sup>13</sup> Bashford, E. F., *4th Scient. Rep. Inv. Imp. Cancer Research Fund*, London, 1911, 131, 173.

tumors were often found to be easily transplantable and might be considered as clinically malignant. However, it cannot be doubted that histological characteristics are related to certain physiological properties. Malignant degeneration manifests itself by simultaneous morphological and functional changes. These changes are quantitative rather than qualitative. They occur gradually. There is no sharp border line between benign and malignant degeneration. The differences in the behavior *in vitro* of explants from various tumors could conceivably be used as a means of diagnosis. By studying the mode and rapidity of cell migration, the digestion of egg albumin, and acid production of fragments of tumors, it has been possible to determine the histological nature of the tissue. It remains to be ascertained whether similar differences between various types of tumors are of general significance. If such is the case, tissue culture might possibly be used not only for making differential diagnosis, as suggested by Kapel,<sup>14</sup> but also for determining the degree of malignancy of a given tumor.

#### SUMMARY

1. The histological and physiological characteristics *in vitro* of mouse tumors have been compared.

2. Twenty-eight adenomas, 31 adenocarcinomas, 18 carcinomas, and a series of tumors from 2 transplantable carcinomas and from Ehrlich carcinoma have been cultivated *in vitro*.

3. The adenomas were characterized by cells of normal appearance, migrating quickly and organizing in thin membranes, by few mitoses, a large acid production, and a rapid digestion of fibrin and egg albumin.

4. The carcinomas differed from adenomas in a less rapid and extensive migration of epithelial cells, a tendency to grow in bud-like formations, the presence of many mitoses, and a lesser acid production and fibrin and albumin digestion.

5. Ehrlich carcinoma differed from both adenomas and spontaneous carcinomas in the unhealthy state of the cells, their irregular growth, and the occurrence of a great many mitoses. Acid production was slight, and digestion of egg albumin or fibrin less marked, and even entirely lacking.

<sup>14</sup> Kapel, O., *Arch. exp. Zellforsch.*, 1929, 8, 35.

6. The adenomas and spontaneous carcinomas did not invade embryonic tissue. The transplantable carcinomas invaded it slightly, while Ehrlich carcinoma actively infiltrated and destroyed it.

## EXPLANATION OF PLATES

## PLATE 47

FIG. 1. Section of spontaneous mammary gland adenoma, fixed and stained with hematoxylin and eosin.  $\times 230$ .

FIG. 2. Section of spontaneous mammary gland adenocarcinoma, fixed and stained with hematoxylin and eosin. Carcinomatous structure at left, adenomatous at right.  $\times 230$ .

FIG. 3. Flask cultures of adenoma (*A*), adenocarcinoma (*B*), and Ehrlich carcinoma (*C*), cultivated in the same flask; one culture from each placed on egg albumin. Note digestion of egg albumin in *A* and *B*, absence of digestion in *C*, and epithelial migration of control explants placed directly in clot.  $\times 5$ .

## PLATE 48

FIG. 4. Living flask culture of adenoma, showing regular epithelial migration.  $\times 28$ .

FIG. 5. Living flask culture of Ehrlich carcinoma cultivated in the same flask with culture in Fig. 4, showing bud-like processes.  $\times 28$ .

FIG. 6. Section of a flask culture of adenoma (*A*), cultivated with embryonic tissue (*F*); fixed and stained with hematoxylin and eosin. No invasion of normal tissue by adenoma.  $\times 328$ .

FIG. 7. Section of a flask culture of Ehrlich carcinoma (*E. C.*), cultivated with embryonic tissue (*F*); fixed and stained with hematoxylin and eosin. Invasion of normal tissue by carcinoma.  $\times 328$ .

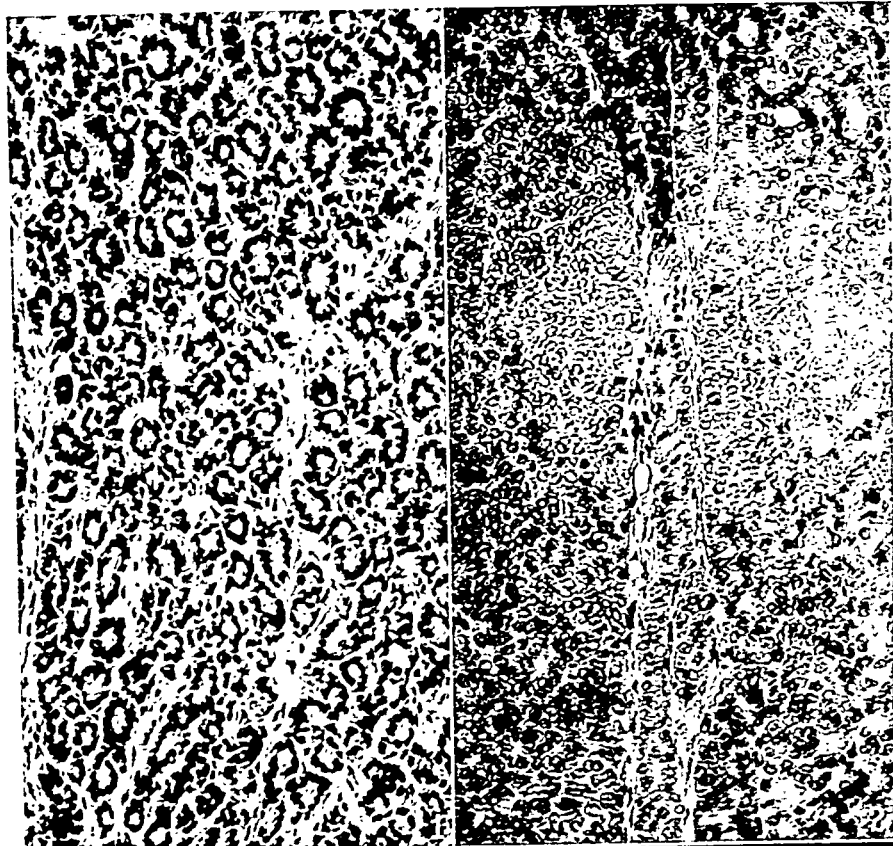
## PLATE 49

FIG. 8. Flask culture of adenocarcinoma, fixed and stained with hematoxylin and eosin. Regular cell formations. No mitoses.  $\times 392$ .

FIG. 9. Flask culture of Ehrlich carcinoma, fixed and stained with hematoxylin and eosin. Crowding of cells. Many mitoses.  $\times 392$ .

FIG. 10. Living flask culture of adenoma on egg albumin. Marked digestion.  $\times 34$ .

FIG. 11. Living flask culture of Ehrlich carcinoma on egg albumin. No digestion.  $\times 34$ .

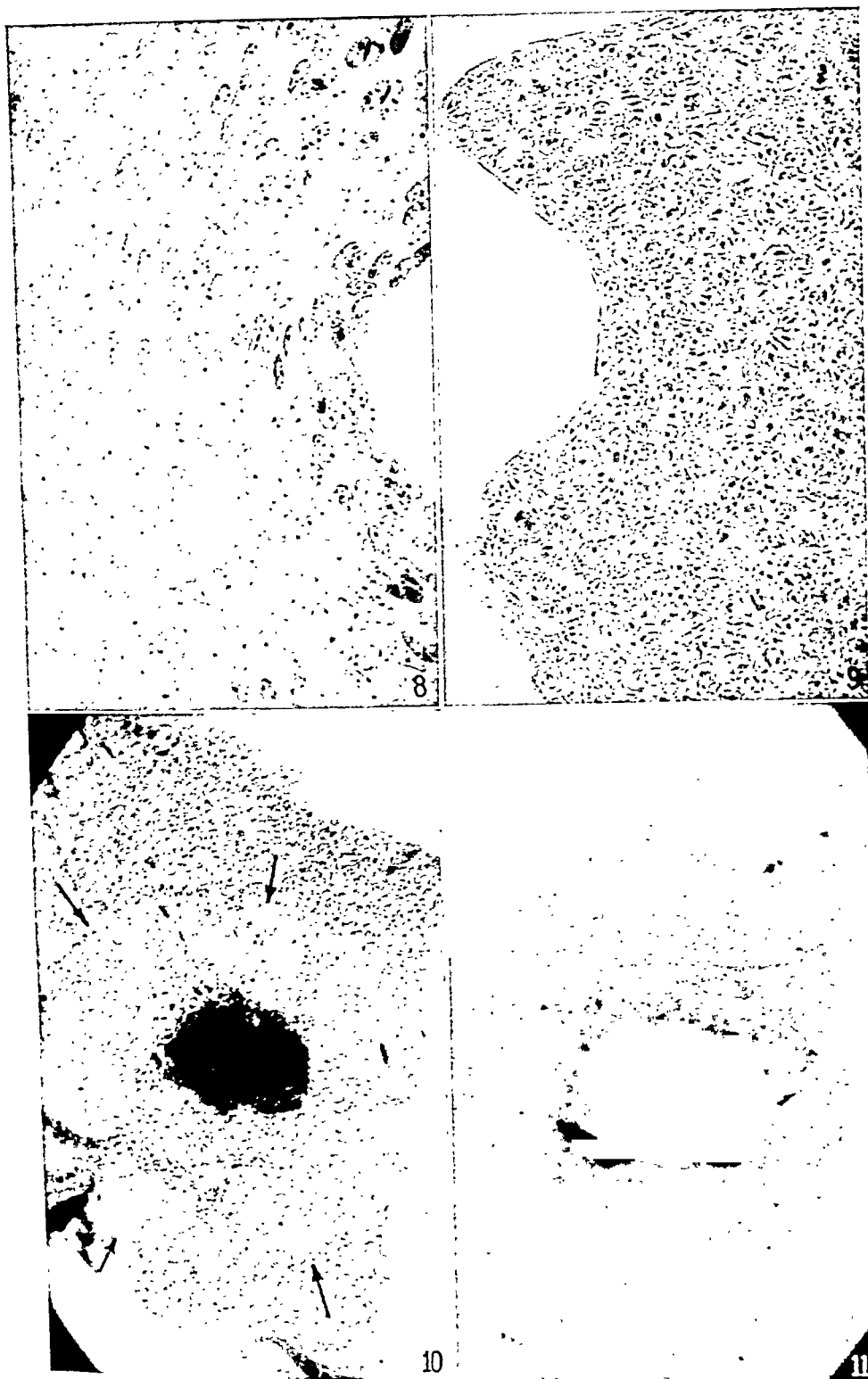






(Sarcomatous Epithelial tumors of the mouse)





(Centers in Epithelial tumors of the mouse)





A STUDY OF THE DISSOCIATION OF THE RAWLINS STRAIN  
OF BACTERIUM TYPHOSUM WITH SPECIAL REFER-  
ENCE TO ITS USE IN THE PRODUCTION OF ANTI-  
TYPHOID VACCINE

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PLATE 50

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The principles upon which prophylactic inoculation against typhoid fever is based are unquestionably sound, and there can be no doubt of the fact that the procedure has considerably diminished the incidence of the disease during the last thirty years. The instances of the failure of antityphoid vaccine to protect against infection are, however, not so rare as to preclude the possibility that some of the vaccines now in use are not as efficient as they should be. Practically all the vaccine used in this country is made from the Rawlins strain of *Bacterium typhosum*—a culture isolated by Sir Almroth Wright in 1903—although our knowledge of bacterial dissociation strongly suggests that such an old culture would by now have become considerably altered in antigenic composition.

That rough strains of practically all the pathogenic microorganisms are inferior to the smooth forms as protective antigens has been demonstrated by many different workers. The results obtained by Weber (1) and by Arkwright (2) with guinea pigs actively immunized with the rough and smooth variants of typhoid and paratyphoid cultures indicate that this rule applies likewise to the enteric group. The report of the British Army Council (3) that non-virulent strains are as suitable as virulent cultures for making antityphoid vaccine was based on the assumption—since proved erroneous—that the ability of a vaccine to produce flagellar agglutinins is synonymous with its protective efficiency.

Whitehead (4), in comparing the Rawlins strain with a freshly isolated smooth culture, although observing certain cultural differences between them, concluded that the Rawlins must be considered a smooth strain because it produced somatic

agglutinins when injected into rabbits. Since, however, when he tested the resistance of his immunized rabbits to the injection of a living, smooth culture, the three Rawlins rabbits died, whereas two of the three smooth vaccine rabbits lived, he expressed a doubt as to whether we are justified in using somatic agglutination to estimate the immunity conferred by a vaccine.

The writer (5) has reported the results of comparative bactericidal tests with the blood of human beings before and after receiving the customary course of prophylactic inoculations with a vaccine prepared from a smooth, freshly isolated strain of *Bacterium typhosum* and with a vaccine made from the Rawlins strain. In these experiments, whereas the bactericidal power of the blood of those receiving the smooth vaccine showed a considerable increase, the bactericidal power of the blood of those receiving the Rawlins vaccine was slightly less after vaccination than before.

The present paper gives the results of a study of a number of Rawlins cultures now in use in this country for making vaccine, and presents a comparison of the protective efficiency of these cultures with that of a number of freshly isolated smooth strains. In order to do this properly, it was necessary to carry out a preliminary study of the cultures employed from the point of view of dissociation, since there has been no generally accepted and precise definition of smoothness and roughness in this group and of the relationship of individual dissociation forms to immunizing power.

For this purpose, twelve Rawlins cultures were obtained through the kindness of the directors of twelve different laboratories engaged in making antityphoid vaccine. These laboratories were in the main selected to represent those whose vaccine is most widely used, although all these cultures originated at one time or another from the cultures maintained at the Army Medical School or at the National Institute of Health in Washington. Four of these strains were considered to be smooth in the laboratories from which they were obtained, two were considered as intermediate and three as rough. On three others no opinion was offered as to their state of dissociation.

The twelve Rawlins cultures were compared with a number of freshly isolated smooth strains in respect to morphology, motility, colony form, nature of growth in infusion broth, growth in 10 per cent normal horse serum broth after 6 hours' and after 24 hours' incubation and stability of suspension in normal saline solution.

### *Cultural and Serological Characteristics*

*Morphology.*—Although the morphological differences between the smooth strains and the Rawlins cultures were not sufficiently distinct to differentiate one from the other with certainty, the Rawlins organisms did show a much greater tendency to appear as long, thread-like forms. Furthermore, when stained with a modification of the Hiss capsule stain, the bacilli of the smooth cultures gave an appearance resembling capsule formation which was not obtained in similar preparations of the Rawlins cultures.

*Motility.*—Five of the Rawlins strains were composed predominantly of motile forms; four were largely non-motile, and three showed no evidence of motility in hanging drop preparations or in cultures in semisolid agar.

*Colony Form.*—It is evident that a number of factors influence the appearance of the colonies on infusion agar or on blood agar plates. Chief among these factors are the moistness or dryness of the plates, the length of incubation time of the cultures and the degree of separation of the colonies on the plate. Under comparable conditions of cultivation, however, two of the Rawlins cultures produced colonies corresponding to the classical rough type; that is, large, flat and rugose, with irregular edges. The other ten cultures showed colonies which were intermediate between this typical rough form and the smaller, shining, lenticular colonies of the smooth cultures.

Fig. 1 shows colonies of a freshly isolated smooth culture. The colonies in Fig. 2 are from a culture which had been isolated 2 years previously and which, although showing a slight roughening of colony surface, had not altered in virulence or in protective efficiency. Fig. 3 shows colonies of the intermediate type from one of the Rawlins cultures and Fig. 4 represents a typical rough colony.

*Growth in Infusion Broth.*—Smooth strains of *Bacterium typhosum* give a uniformly turbid growth in infusion broth. Three of the less motile Rawlins cultures showed this smooth type of growth. Two cultures flocculated completely, leaving a clear supernatant fluid; i.e., the typical rough manner of growth. Seven cultures were turbid but with a heavy sediment and a distinct pellicle.

*Growth in 10 Per Cent Normal Horse Serum Broth.*—In 10 per cent normal horse serum broth smooth cultures are uniformly turbid from the time growth becomes readily visible. The twelve Rawlins cultures, however, showed a flocculent type of growth up to about 6 hours' incubation. After 24 hours two of these cultures were turbid with a heavy sediment, eight were turbid with sediment and pellicle and two were still flocculated.

*Stability of Suspensions in Normal Saline Solution.*—Eleven of the Rawlins strains formed stable suspensions in 0.85 per cent sodium chloride solution, as do typical smooth cultures. One strain, which gave the other reactions of a rough variant, while still suspended after 2 hours in the water bath, sedimented after remaining a further 12 hours in the refrigerator.

*Agglutination of the Rawlins Cultures by Anti-Smooth Sera.*—Agglutination tests

were carried out with the twelve Rawlins cultures, using the serum of rabbits immunized with a motile, smooth and with a non-motile, smooth culture. With the anti-motile, smooth serum eight of the Rawlins suspensions showed the flocculent, flagellar type of agglutination in a dilution of 1/12,800, which was also the titre of this serum against a motile, smooth culture. The three non-motile cultures gave the granular, somatic type of agglutination in a serum dilution of 1/6,400, and one culture failed to agglutinate in a 1/100 dilution of this serum. The serum of the rabbit immunized with the non-motile, smooth culture agglutinated eleven of the Rawlins strains in dilutions varying from 1/200 to 1/3,200, which was the titre of this serum against smooth cultures. The culture which failed to agglutinate in the anti-motile, smooth serum also gave no agglutination in the anti-non-motile smooth serum.

### *Comparison of the Virulence of the Rawlins Cultures with the Virulence of Smooth Strains*

The virulence of the twelve Rawlins cultures was now compared with the virulence of a number of freshly isolated strains, by determining the number of organisms killed by a given quantity of fresh, defibrinated guinea pig blood and by virulence tests in mice.

*Bactericidal Tests.*—In the bactericidal tests a series of tubes containing 0.5 cc. of a 1/4 dilution of fresh, defibrinated guinea pig blood was inoculated with 1 drop of progressive dilutions of an 18 hour broth culture of the organism to be tested. The tubes were then sealed and incubated in a rotating box to insure thorough mixing. After 48 hours' incubation, a loopful of each tube was plated out to determine whether growth had taken place. In such tests the 0.5 cc. of diluted fresh guinea pig blood failed to kill the ten or fifteen smooth organisms contained in 1 drop of the  $10^{-6}$  dilution of culture. In other words, this dilution of fresh guinea pig blood is not bactericidal for smooth typhoid bacilli. On the other hand, approximately 100,000 organisms of the Rawlins cultures contained in 1 drop of the  $10^{-2}$  dilution were killed by the same quantity of diluted guinea pig blood which had no bactericidal effect on the smooth cultures.

*Virulence Tests in Mice.*—Preliminary virulence tests in mice with smooth strains of *Bacterium typhosum* showed that the percentage mortality decreased very slowly as the dose was reduced; namely, 0.1 cc. of an 18 hour broth culture killed 95 per cent, 0.01 cc. killed 60 per cent and 0.001 cc. killed 20 per cent of the mice injected. It was impossible with any degree of accuracy, therefore, to express the difference in the virulence of various strains as a difference in the minimum lethal dose. The percentage mortality resulting from the injection of the 0.1 cc. dose was, therefore, used to express the difference in virulence of the Rawlins and the smooth cultures. In such tests five mice were injected with each of the twelve Rawlins cultures. Each mouse received intraperitoneally 0.1 cc. of an 18 hour broth culture so diluted that the dose was contained in 0.5 cc. Of the 60 mice used

in this test only two, or 3.3 per cent, died. In contrast to this, six smooth cultures killed all but two of the 40 mice similarly tested—a mortality of 95 per cent.

### *Active Immunization of Mice*

Three preliminary experiments were carried out to test the immunity developed in mice following active immunization with vaccines prepared from one of the Rawlins cultures and from a smooth culture. The method of immunization employed in these three experiments varied somewhat and the test doses were given in both 0.1 cc. and 0.01 cc. amounts. As the results, however, were essentially similar, they may be summarized as follows: Of sixteen control mice, fourteen, or 87 per cent died; of thirty-two Rawlins vaccine mice, twenty-eight, or 87 per cent died; while of thirty-one mice receiving the smooth vaccine, only one, or 3 per cent died.

Since the results of these mouse protection tests agreed with the previously reported experiments on the effect of the Rawlins and the smooth vaccine on the bactericidal power of human blood (5), mouse immunization was considered a fairly adequate and simple method of comparing the protective efficiency of different strains of typhoid bacilli. Five mice were therefore immunized with each of the twelve Rawlins strains, and the mortality following the injection of a living, smooth culture compared with that of a number of control mice immunized with smooth vaccines.

The procedure was as follows: Each mouse was given two injections at 5 day intervals of 0.1 cc. of a 24 hour broth culture killed by heating at 56°C. for 1 hour. 10 days after the second injection, they received as the test dose 0.1 cc. of an 18 hour broth culture of a smooth strain—a dose which, as previously noted, resulted, in untreated mice, in a mortality of 95 per cent. Of the 60 mice vaccinated with the twelve Rawlins vaccines, 55, or 91.6 per cent died, whereas of thirty-seven mice similarly vaccinated with six different smooth cultures and tested as controls, only two, or 5.4 per cent died.

In addition to the twelve cultures of the Rawlins strain used in the above studies, one laboratory sent in, as the culture they are now using for vaccine, a strain isolated from the blood of a typhoid patient in Nov., 1931. This strain gave all the cultural and serological reactions of a motile, smooth typhoid bacillus. It grew out in the 1/4 dilution of fresh, defibrinated guinea pig blood through the 10<sup>-6</sup> dilution; 0.1 cc. of an 18 hour broth culture killed the five mice tested, and in the mouse protection test four of the five mice survived. (The fifth mouse did not appear well at the time the test dose was given.)

As a final comparison of the Rawlins and the smooth vaccine, five mice were given two intraperitoneal injections, each at an interval of 5 days, of 0.1 cc. of the vaccine prepared by one of the laboratories from what was considered by them to be a smooth Rawlins culture. Five mice were given two similar injections each of the vaccine prepared by the laboratory using the recently isolated smooth culture. 10 days after the second injection these ten mice each received 0.1 cc. of an

*Cultural, Serological and Immunological Reactions*

Culture	Motility	Colony form	Growth in broth	
A Rawlins	Non-motile	Intermediate	Turbidity with pellicle and sediment	(
B Rawlins (smooth)†	Predominantly motile	Intermediate	Turbidity with pellicle and sediment	C
C Rawlins (smooth)†	Non-motile	Intermediate	Uniform turbidity	C
D Rawlins (rough)†	Non-motile	Intermediate	Uniform turbidity	Co 1
E Rawlins (intermediate)†	Predominantly non-motile	Intermediate	Uniform turbidity	Co fi
F Rawlins	Predominantly motile	Intermediate	Turbidity with pellicle and sediment	Cor flo
G Rawlins (rough)†	Predominantly motile	Intermediate	Turbidity with pellicle and sediment	Com flo
H Rawlins (smooth)†	Predominantly motile	Intermediate	Turbidity with pellicle and sediment	Com floc
I Rawlins (smooth)†	Predominantly non-motile	Intermediate	Turbidity with pellicle and sediment	Comp floc
J Rawlins (intermediate)†	Predominantly motile	Intermediate	Turbidity with pellicle and sediment	Compl floc
K Rawlins	Predominantly non-motile	Rough	Completely flocculated	Comple flocc
L Rawlins (rough)†	Predominantly non-motile	Rough	Completely flocculated	Comple floccu
M§ Freshly isolated smooth (smooth)†	Predominantly motile	Smooth	Uniform turbidity	Uniform bidity

\*  $10^{-1}$  indicates that growth took place only in the tube inoculated with 1 drop of the  $10^{-1}$  dilution.  
 $10^{-2}$  indicates that growth took place in the tube inoculated with 1 drop of the  $10^{-1}$  dilution and  
 $10^{-6}$  indicates that growth took place in all tubes, including that inoculated with 1 drop of the  $10^{-1}$  dilution.

† See text for method. Five mice used in each test.

‡ Opinion of laboratory submitting culture.

§ Five other recently isolated strains.

*Lines of Bacterium typhosum Used for Vaccine Production*

Normal horse serum broth 24 hrs.	Suspension in normal saline solution	Agglutination		Growth in normal guinea pig blood*	Virulence for mice†	Mouse protection test‡
		Anti-MS serum	Anti-NS serum			
Turbidity with pellicle and sediment	Stable	1/6,400 Granular O	1/3,200 Granular O	10 <sup>-1</sup>	5 survived	5 died
Turbidity with pellicle and sediment	Stable	1/12,800 Flocculent H	1/800 Granular O	10 <sup>-1</sup>	5 survived	5 died
Turbidity with sediment	Stable	1/6,400 Granular O	1/3,200 Granular O	10 <sup>-1</sup>	5 survived	5 died
Completely flocculated	Stable	1/6,400 Granular O	1/3,200 Granular O	10 <sup>-1</sup>	5 survived	4 died
Turbidity with pellicle and sediment	Stable	1/12,800 Flocculent H	1/400 Granular O	10 <sup>-1</sup>	5 survived	4 died
Turbidity with pellicle and sediment	Stable	1/12,800 Flocculent H	1/800 Granular O	10 <sup>-1</sup>	5 survived	5 died
Turbidity with pellicle and sediment	Stable	1/12,800 Flocculent H	1/800 Granular O	10 <sup>-2</sup>	3 survived	5 died
Turbidity with pellicle and sediment	Stable	1/12,800 Flocculent H	1/200 Granular O	No growth in 10 <sup>-1</sup>	5 survived	5 died
Turbidity with pellicle and sediment	Stable	1/12,800 Flocculent H	1/800 Granular O	10 <sup>-1</sup>	5 survived	3 died
Turbidity with sediment	Stable	1/12,800 Flocculent H	1/400 Granular O	10 <sup>-1</sup>	5 survived	4 died
Completely flocculated	Unstable	Negative	Negative	10 <sup>-1</sup>	5 survived	5 died
Turbidity with pellicle and sediment	Stable	1/12,800 Flocculent H	1/1,600 Granular O	10 <sup>-1</sup>	5 survived	5 died
Uniform turbidity	Stable	1/12,800 Flocculent H	1/3,200 Granular O	10 <sup>-2</sup>	0 survived	1 died

\* 18 hour broth culture.

† That inoculated with 1 drop of the 10<sup>-2</sup> dilution of an 18 hour broth culture.

‡ None.



## RAWLINS STRAIN OF BACTERIUM TYPHOSUM

18 hour broth culture of a heterologous smooth strain. Three of the mice immunized with the Rawlins vaccine died, while the five smooth vaccine mice survived. Table I shows the cultural, serological and immunological reactions of the twelve Rawlins cultures compared with those of smooth strains.

## DISCUSSION

The changes which take place in morphological and cultural characteristics owing to bacterial dissociation are of great theoretical interest; but it is the alterations in virulence and in antigenic composition which are of major practical importance.

The Rawlins strain of *Bacterium typhosum* isolated by Sir Almroth Wright in 1903 is almost universally employed for making vaccine in this country as well as in England, and has almost attained the status of an official typhoid culture. There is no doubt that this culture was originally and still is a typhoid bacillus; but this does not mean that thirty years of life on artificial media have not led to a degree of dissociation which has materially altered its antigenic constituents.

The present study, which was undertaken to determine the dissociation form of the cultures actually in use for vaccine production in this country and to compare the protective efficiency of these strains with that of freshly isolated, smooth cultures, showed that one of these representative Rawlins strains corresponded in cultural and serological characteristics to the classical rough variant. The remaining eleven cultures gave cultural reactions intermediate between this typically rough strain and typically smooth cultures. In the stability of their suspensions in normal salt solution and in their agglutinability in the serum of rabbits immunized with non-motile, smooth antigens—criteria on which Arkwright lays great stress—they corresponded to the smooth phase; but as judged by the more significant tests of resistance to the bactericidal action of normal blood and by their virulence for mice, they should be considered rough.

Since these twelve representative subcultures of the Rawlins strain all differed materially from the smooth cultures with which they were compared, it would seem safe to assume that any laboratory using this organism for vaccine production is not using a typical smooth typhoid bacillus. From the point of view of vaccine production, however, it is of much more practical importance to determine the degree

of protection against typhoid fever conferred by the Rawlins strain than to discuss whether it is a smooth, intermediate or rough variant.

Three practicable methods are at hand for testing the antigenic potency of a vaccine: namely, agglutination by the serum of vaccinated animals or man; bactericidal tests with human blood following vaccination, and protection tests with actively immunized animals. The direct test of protection in man is of course out of the question.

It has been demonstrated by many workers that the heat-labile, flagellar H antigen is the same in the smooth and the rough phase, and that the flocculent H agglutinins are not a measure of protection. Nevertheless, it is still a common practice, when such tests are made, to assess the potency of a vaccine by its ability to induce the formation of this H type of agglutinin, and to judge the protection of vaccinated individuals by the same test.

It has been maintained by numerous workers in this field that the heat-stable, somatic O substance is characteristic of the smooth variants, and that O agglutinins develop *pari passu* with bactericidal and protective antibodies. On this basis, one would assume any culture giving the somatic type of agglutination with anti-smooth serum to be a smooth culture, and that the presence of O agglutinin in a serum was evidence of immunity. The present study shows, however, that certain intermediate forms of the Rawlins strain, while giving the somatic type of agglutination with anti-smooth sera, were in cultural characteristics and in virulence quite different from the smooth form. Furthermore, the human immunization experiments previously reported and the mouse protection tests here presented show that these cultures, while inducing the formation of O agglutinins in man and rabbits, did not increase the bactericidal power of human blood, nor protect mice against subsequent infection with smooth cultures.

Agglutination, therefore, of neither the flagellar nor the somatic type can be used as a test of the protective power of a vaccine.

The bactericidal test, while obviously open to the objection that it deals only with the serum antibodies<sup>1</sup> and leaves out of consideration the possibly very important changes in fixed cell reaction following prophylactic vaccination, offers the most direct practicable method of

<sup>1</sup> We know from other experiments that the circulating leucocytes play little or no rôle in intravascular protection against *Bacterium typhosum*.

testing the effect of immunization in man. Compared by this method a vaccine prepared from a Rawlins strain which was considered to be a smooth culture in the laboratory using it for vaccine production proved far inferior to a vaccine made from a freshly isolated strain in its ability to induce the formation of bactericidal antibodies, although it produced a decided rise in the O agglutinins.

Although due consideration must be given the possibility of error in applying directly to man the results of animal experimentation, protection tests in actively immunized mice can be used to supplement the human bactericidal tests, since by this method we can determine the effect of the vaccine on the complete body mechanism of resistance. Such protection tests in mice comparing the antigenic efficiency of the twelve Rawlins cultures with that of six different smooth strains agreed with the bactericidal experiments in showing the inferiority of the Rawlins vaccines. The bactericidal test with the blood of vaccinated human beings and the mouse protection test together undoubtedly constitute a more valid measure of the prophylactic value of a vaccine than does agglutination; and the mouse protection test alone appears to offer a simple and adequate method of assuring the potency of any lot of vaccine.

There is no valid reason to believe the Rawlins strain antigenically superior to other typhoid cultures. Our knowledge of bacterial dissociation would lead us to suspect that this very old culture had by now assumed other than the smooth form. This suspicion is borne out by a study of the cultural characteristics and virulence of twelve subcultures of the strain. Bactericidal tests with the blood of vaccinated human beings and protection tests with mice indicate that the Rawlins culture is far inferior to smooth strains as a protective antigen.

It might seem that difficulty would arise in the selection and maintenance of a suitable smooth culture for use in vaccine production. From the report of Weber (1), however, on cross-protection tests with smooth strains, and from the results of similar tests with six different smooth cultures in the present series, it would appear that the typhoid bacillus is a homogeneous organism—so far, at least, as its protective antigens are concerned—and it is therefore a matter of small importance what strain is selected, as long as it is strictly smooth. Furthermore, since this organism shows relatively little tendency to dissociate

into the rough variant, there is no need to resort to frequent fresh isolations, or to animal passage in order to insure the use of a smooth culture. We have in our laboratory a strain which has been kept on agar slants and transferred every 2 weeks for 3 years without loss of virulence or protective efficiency.

Since the use of smooth cultures for vaccine presents no serious difficulty and since the most direct practicable tests of their protective value indicate their decided superiority, while tradition alone supports the use of the Rawlins strain, it would appear practically certain that the use of smooth cultures would very considerably increase the prophylactic efficiency of antityphoid vaccines.

#### CONCLUSIONS

A study of the subcultures of the Rawlins strain of *Bacterium typhosum* used by twelve different laboratories for vaccine production showed that they all differed from recently isolated smooth strains in cultural characteristics, virulence and protective efficiency.

Eleven of these Rawlins cultures gave both the flagellar and the somatic type of agglutination in anti-smooth rabbit serum, and the one culture so tested produced both flagellar and somatic agglutinins when injected into rabbits and man.

Agglutination of neither the flagellar nor the somatic type can, therefore, be used as a test of the smoothness of a culture or as an index of immunity.

Since the Rawlins strain differs from the smooth phase of *Bacterium typhosum* in cultural characteristics and in virulence, and is much less efficient than smooth strains as a protective antigen; and since the selection and maintenance of smooth cultures suitable for vaccine production present no serious difficulty, it would seem but logical to substitute virulent, smooth cultures for the very old Rawlins strain, if we are to expect the maximum protection from antityphoid vaccination.

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## RAWLINS STRAIN OF BACTERIUM TYPHOSUM

## EXPLANATION OF PLATE 50

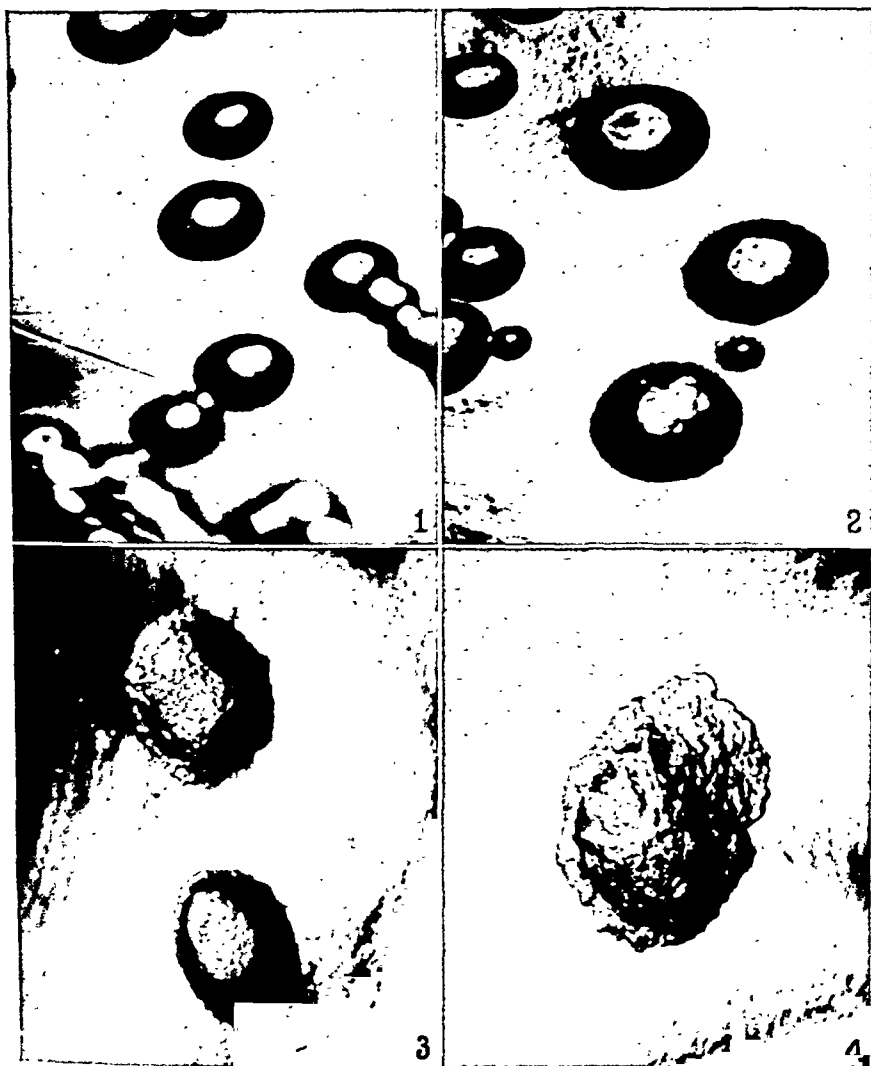
All colonies are from 12 hour cultures on 5 per cent horse blood agar.  
The photographs were taken by Dr. C. V. Seastone.

FIG. 1. Smooth colonies from a freshly isolated strain of *Bacterium typhosum*.  
× 14.

FIG. 2. Slightly rough colonies from a culture of *Bacterium typhosum* which had been under artificial cultivation for 2 years. Except for the slight roughening of the colony surface, this culture was still smooth. × 14.

FIG. 3. Intermediate type of colony from a culture of the Rawlins strain. × 14.

FIG. 4. Colony from a typical rough culture of the Rawlins strain. × 14.

(Grinnell-Rawlin strain of *Bacterium typhosum*)



# THE EXTRACARDIAC ANASTOMOSES OF THE CORONARY ARTERIES

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PLATES 51 TO 53

(Received for publication, July 29, 1932)

In a series of experiments planned for the purpose of injecting the vessels in the heart valves, a colloidal suspension of carbon particles was injected into the coronary arteries of human hearts which had been excised at autopsy. When one came to study the injected specimens it was soon observed not only that the vessels in the heart were filled with the carbon particles but also that the arteries in attached flaps of the parietal pericardium contained the injection mass. Further observation showed that an extensive network of vessels in the adventitia of the aorta and pulmonary artery was also injected.

These observations led to a more thorough study of the extracardiac anastomoses of the coronary arteries. The results of this investigation are reported here.

The literature furnishes little if any detailed description of the extracardiac anastomoses of the coronary arteries. The most instructive paper is that of Langer (4) who, during a study of the Thebesian vessels by coronary artery injection, described branches of the coronary arteries anastomosing with vessels in the mediastinum, the parietal pericardium, the diaphragm and the hila of the lungs. Woodruff (10) in 1926 called attention to anastomoses of branches of the coronary arteries with the vasa vasorum of the ascending aorta. In 1928 Wearn (9) noted that on injecting the coronary arteries with India ink or Berlin blue the vessels in the ascending aorta were filled with the injection mass. Robertson (6) in 1930 pointed out the importance of the arteries of the fat pads of the heart in the presence of disease of the coronary arteries. He discussed also the anastomoses of branches of the coronary arteries with the periadventitial vessels of the ascending aorta and other vessels of the thorax. Several reviews of the literature including those of Gross, 1921 (2), Mönckeberg, 1924 (5), Spalteholz, 1924 (7),



Warburg, 1930 (8) and Karsner (3), have mentioned the extracardiac coronary anastomoses.

The exact extent and origin of the extracardiac anastomoses of the coronary arteries with the arteries of the nearby tissues and structures have not been clearly defined or described. Even their presence is not generally known. In view of this fact and with the possibility in mind that these anastomoses may be of important physiological and pathological significance, this investigation was undertaken.

#### EXPERIMENTAL

The injection method employed was that described by Bromer, Zschiesche and Wearn (1). Glass cannulae were inserted and tied into the coronary arteries from within the aorta through a longitudinal slit in the aortic wall. The injection mass used was a 3 per cent suspension of lamp black and 5 per cent acacia in distilled water. In some instances India ink diluted with an equal part of distilled water was used and found satisfactory. The injection mass was warmed to 40°C. and specimens were injected in a water bath at about the same temperature and massaged during injection. In a few experiments a suspension of 10 per cent bismuth oxychloride and 6 per cent acacia (approximately) in distilled water was used instead of the carbon suspension.

The cannulae were connected by short pieces of rubber tubing to a Keyes two-way stop-cock so that either negative or positive pressure could be used. At the beginning of the experiment, suction was applied for a few minutes while the heart was being massaged, to withdraw air and blood from the vessels. The mass was then injected under a positive pressure of 220 mm. of mercury for 5 minutes or longer.

The extent of the anastomoses demonstrated depended in some degree upon the type of specimen injected. In some cases the coronary arteries were injected with the heart *in situ*. This was accomplished after removal of the anterior thoracic wall by cannulating the coronary arteries through a small incision in the aorta. The surrounding structures were disturbed as little as possible. Satisfactory injections of specimens were obtained, however, in eviscerated preparations, where the thoracic organs, the diaphragm and the abdominal viscera were removed *en bloc*, and care was taken to keep the pericardium intact around the openings of the great veins. The presence of intact pericardium around the root of the aorta and pulmonary artery was not so essential to a satisfactory extracardiac injection as was the presence of undamaged pericardial reflections around the great veins.

After the injection was completed, the vessels were described and sketched and the entire specimen was placed in 10 per cent formalin for from 24 to 48 hours. Blocks were cut and sections prepared to demonstrate the presence of intravascular

injection mass microscopically. Thirty-one specimens were injected in this manner, of which number, twelve consisted of heart and parietal pericardium; eight of heart, parietal pericardium and diaphragm; and five of heart, parietal pericardium, lungs and diaphragm. Four others were the eviscerated thoracic organs without any dissection and two were injected *in situ*.

Careful study of the injected material revealed extensive anastomoses between the coronary arteries and the vessels of the neighboring organs and other structures. These extracardiac branches of the coronary arteries emerged from the heart in several regions and were constant in the material studied, no variations of moment being noted. Among the more common sites of emergence were (1) around the root of the aorta; (2) around the base of the pulmonary artery; (3) around the pulmonary veins; (4) around the ostia of the superior and inferior venae cavae, and (5) in the intervascular pericardial reflections. A more detailed description of these vessels follows:

1. The vessels emerging around the root of the aorta were direct branches of the coronary arteries arising near the aorta and extensions of the branches to the pericardial fat pads into the adventitia of the aorta. The largest injected vessels were found just above the aortic valve on the anterior surface of the aorta. The vasa vasorum within the wall of the aorta, as well as the arteries and arterioles in the adventitial and periadventitial connective tissue, were injected regularly from the aortic ring to the diaphragm and occasionally for a short distance below the diaphragm. These vasa vasorum were also injected in part through anastomoses with the pericardial, mediastinal and diaphragmatic vessels.

2. The vessels emerging at the base of the pulmonary artery were either within the wall of or closely adherent to the artery and its branches. They maintained these positions even after the branches were well within the substance of the lungs. It was noted that these vasa vasorum of the pulmonary artery received their injection mass from several sources; namely, directly through coronary branches from the conus (Fig. 4), from pericardial reflections around the pulmonary artery, from the injected mediastinal and bronchial arteries and from the arteries in the subpericardial fat pads at the base of the heart.

3. Many arteries were found leaving the heart around the pulmonary veins. They emerged as a rule at the site of the pericardial re-

flexions around these veins. As shown by the injection mass they formed anastomoses with branches of the pericardial, bronchial and mediastinal arteries. Their importance was indicated by the fact that even when the parietal pericardium was cut away from the aorta, pulmonary artery and both venae cavae, an extensive mediastinal injection was obtained through these perivenous coronary anastomoses, while only a scant extracardiac injection was obtained after the pericardium was cut away from the great veins entering the heart.

4. Larger and more numerous injected vessels were found leaving the heart around the ostia of the superior and inferior venae cavae than were observed around the large arteries. Their course and number were not constant but they were usually closely applied to the adventitia of these veins. Some of the most direct and largest branches of the coronary arteries responsible for the passage of the injection mass to extracardiac structures were the auricular branches of the right coronary arteries. These usually consisted of two branches from the proximal portion of the right coronary artery which arborized in the vicinity of the ostia of the venae cavae, after supplying the right auricular wall. The larger and more constant of the two formed an annulus of fine branches around the ostium of the superior vena cava. The cardiac distribution of these auricular branches of the right coronary artery has been clearly described by Gross. In four hearts in this series in which a radiopaque injection mass was employed and roentgenograms of the hearts were made after injection, the course and origin of these vessels corresponded to Gross' description. Although these auricular branches were usually the largest direct vessels concerned in the extracardiac anastomoses, the arborization of many smaller auricular branches of the coronary arteries around the openings of the pulmonary veins and in the intervascular reflections of the parietal pericardium constituted a more important source for the extracardiac injection. The passage of the injection mass into the vasa vasorum of the aorta and pulmonary artery was principally by means of the rami telae adiposae, although an artery of larger calibre deep in the musculature of the conus and apparently representing a direct coronary branch occasionally supplied the walls of these vessels. Generally, the more complete extracardiac injections were obtained in specimens from persons of advanced years,

which is in accord with the observations of Gross and of Robertson. They found that the arterial branches to the pericardial fat increased with age.

5. No large branches of the coronary arteries were seen leaving the heart through the intervacular reflections of the parietal pericardium. Small vessels were injected, but it appeared that the significant extracardiac coronary branches emerged from the heart around the great vessels rather than between them.

The distribution of the vessels injected through the extracardiac coronary anastomoses was as follows:

There was an extensive network of injected vessels over the entire surface of the parietal pericardium. The largest arteries injected were the pericardiophrenic branches of the internal mammary arteries, which accompanied the phrenic nerves on either side. The small anterior branches of the thoracic aorta also contributed many branches to the lateral and posterior surfaces of the parietal pericardium and these were filled regularly with injection mass. The principal anastomoses of the pericardial vessels with branches of the coronary arteries appeared to be where the pulmonary veins and the venae cavae passed through the pericardium.

In the diaphragm, injected vessels spread out in a fan-shaped manner over each dome from the foramen quadratum. Their anastomoses with the coronary system were apparently through small vessels in the adventitia of the inferior vena cava and by means of the injected terminations of the pericardiophrenic arteries.

Injection mass entered vessels in the hila of the lungs through arteries originating from the auricular branches of both coronary arteries and the branches to the pericardial fat, and extending out along the pulmonary arteries and veins. The injected vessels were found over the pleural surfaces of the lungs and along the bronchi (Fig. 6) where they were seen in the mucosa, even after the second bifurcation.

The mediastinum, trachea and esophagus were richly supplied with vessels which were found to be filled with carbon particles after injection of the coronary arteries. These vessels were principally small anterior branches of the thoracic aorta which anastomosed freely with the bronchial, phrenic, intercostal and branches of the internal mammary arteries. Many vessels in the mediastinum and in sur-

rounding organs contributed branches which anastomosed with those from the coronary arteries. These were, in their usual order of prominence: the pericardiacophrenic branches of the internal mammary arteries and the anterior mediastinal, pericardial, bronchial, superior and inferior phrenic, intercostal and esophageal branches of the aorta (Fig. 5).

In order to determine whether the heart could be injected directly through the thoracic branches of the aorta, a large glass cannula was inserted in the aorta above the coronary orifices. The aorta was tied between the cannula and the heart and also at the level of the diaphragm. The innominate, left common carotid and left subclavian arteries were tied. Injection of the aorta was then made. Microscopic examination of sections of the wall of the left auricle near the openings of the pulmonary veins showed injection mass in the coronary vessels (Fig. 3). The extent and capacity of these channels are now being studied in an attempt to determine the actual part they play in the circulation of the heart in normal and abnormal states.

#### SUMMARY

Widespread anastomoses of the auricular branches and the coronary branches to the pericardial fat with the pericardiacophrenic branches of the internal mammary arteries and the anterior mediastinal, pericardial, bronchial, superior and inferior phrenic, intercostal and esophageal branches of the aorta have been described. The most extensive anastomoses between the cardiac and extracardiac vessels are around the ostia of the pulmonary veins. It was possible not only to demonstrate the passage of injection mass from the coronary arteries into the vessels of surrounding structures, but also to show vessels in the heart injected through the thoracic branches of the aorta.

This rich potential extracardiac coronary collateral circulation is probably of significance in compensating for sclerosis of the large trunks of the coronary arteries.

These experiments were carried out during the course of another study. The authors wish to express their thanks to Dr. E. S. Orgain for taking part in some of the experiments.

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## EXPLANATION OF PLATES

## PLATE 51

FIG. 1. Drawing of heart, lungs and diaphragm after injection of the coronary arteries with a colloidal suspension of carbon particles. A network of injected pericardial and mediastinal vessels is seen. There are large injected pleural and diaphragmatic vessels. About one-half natural size.

## PLATE 52

FIG. 2. Drawing of the inner surface of the parietal pericardium and the inferior surface of the diaphragm. The coronary arteries were injected and the heart removed to expose the sites of anastomoses between coronary and extracardiac vessels. About one-half natural size.

## PLATE 53

FIG. 3. Wall of left auricle with vessels containing carbon particles. The injection was made into the thoracic branches of the aorta and the injection mass entered the heart through the anastomoses around the ostia of the pulmonary veins.  $\times 14$ .

FIG. 4. Longitudinal section of the pulmonary artery and valve of a child. Many large adventitial vessels were injected from the coronary arteries.  $\times 5$ .

FIG. 5. Mucosa of esophagus containing vessels injected with carbon particles from the coronary arteries.  $\times 70$ .

FIG. 6. Mucosa of trachea containing vessels injected with carbon particles from the coronary arteries.  $\times 90$ .

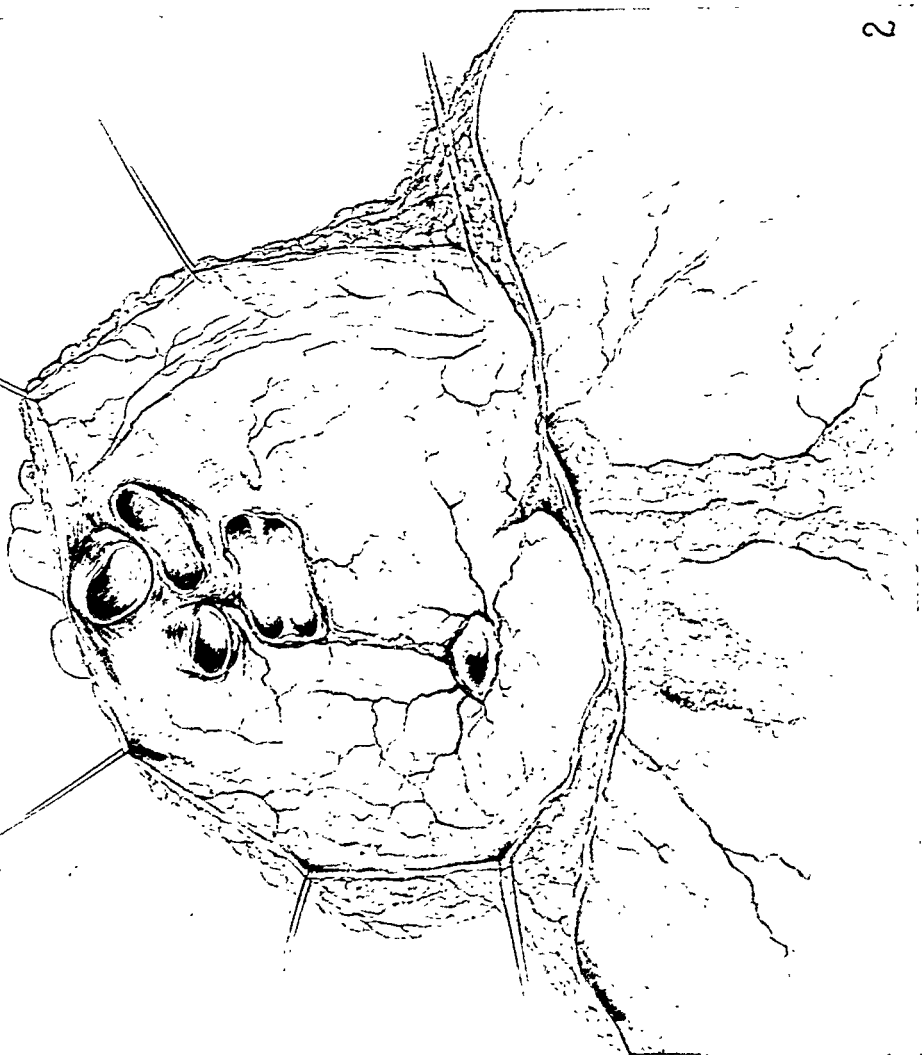




(Hudson *et al.*: Anastomoses of the coronary arteries)

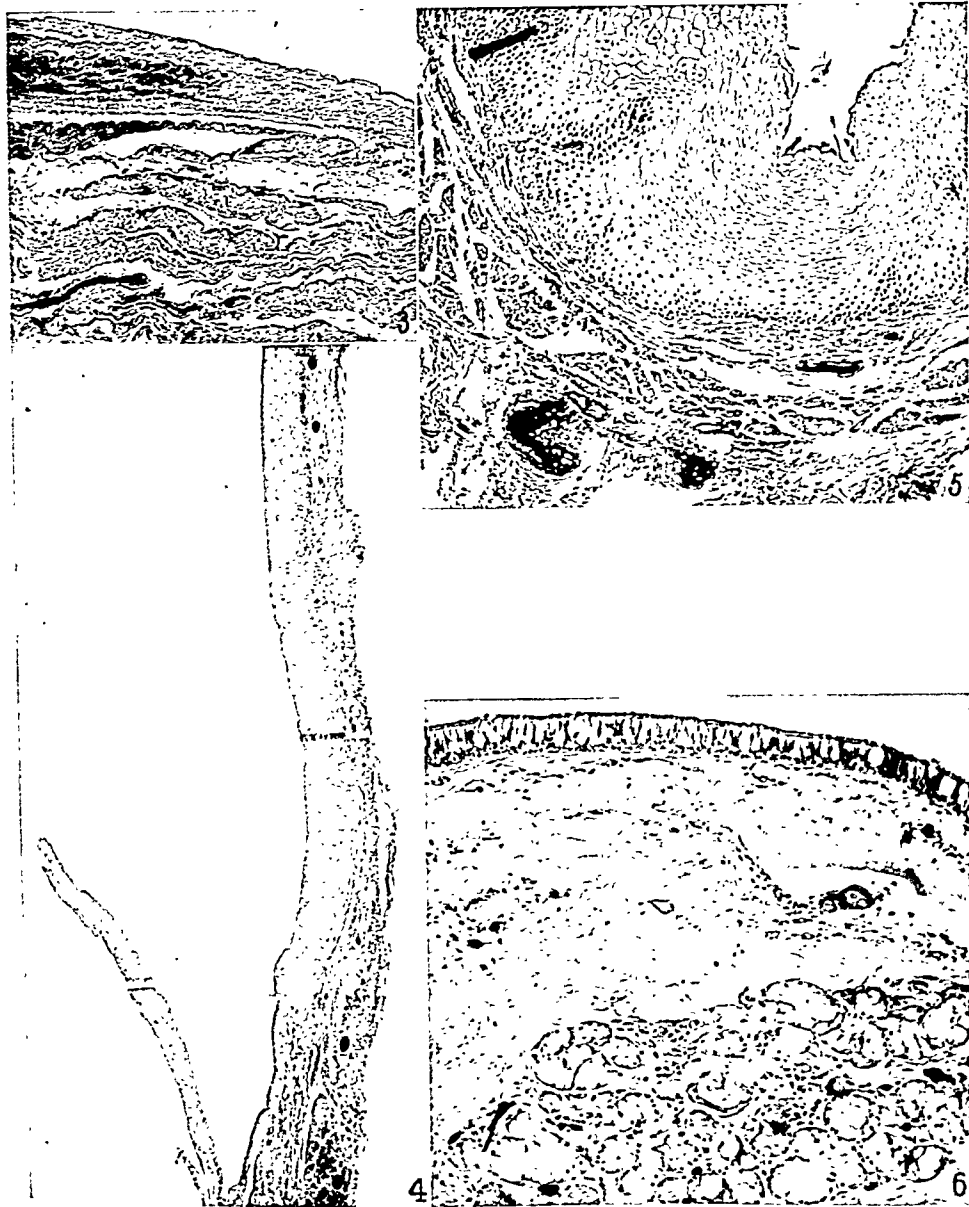






(Hudson *et al* : Anastomoses of the coronary arteries)







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# AUGMENTATION OF THE EXTRACARDIAC ANASTOMOSES OF THE CORONARY ARTERIES THROUGH PERI- CARDIAL ADHESIONS

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PLATE 54

(Received for publication, July 29, 1932)

In the preceding paper, the authors (3) have described the origin and distribution of the anastomoses of the coronary arteries with the vessels of the other thoracic structures. The extent of the participation of this collateral circulation in the event of coronary sclerosis is hypothetical, but it seems entirely probable that such occurs and is at least in part responsible for the survival of individuals in whom both coronary arteries have become occluded at their ostia. A satisfactory demonstration of the correctness of this hypothesis would rest upon arterial injection studies in such cases and it is to be hoped that such investigations will be carried out.

Redwitz (5) and Crooke (1) each have reported cases of bilateral occlusion of the coronary orifices in which they observed a network of dilated vessels extending from the adventitia of the aorta and pulmonary artery to the non-occluded portions of the coronary arteries. Gross (2) suggested the importance of the extracardiac anastomoses in a case of subtotal occlusion of the right coronary artery without infarction, in which the development of the vascularized fat on the right side of the heart was especially prominent. He felt that the coronary branches to the pericardial fat served as a "compensating structure whose functional possibilities increase in direct proportion with age; that is, with that time of life when pathological processes would make an increasing, more frequent and greater demand upon it." This conclusion is directed at the usefulness of the rami telae adiposae in serving as collaterals between the two coronary arteries, but since they are also important, in providing anastomoses between the coronary arteries and the vessels of surrounding structures (Langer (4), Robertson (6), Woodruff (8), Hudson, Moritz and Wearn (3)), an even greater importance may be attached to an increase in their numbers.

The natural corollary to the demonstration of the extracardiac coronary anastomoses would be that whatever vascular reserve such a collateral circulation might provide would be increased in the event of vascularized adhesions between the pericardial surfaces. Thorel (7) reported a case of a man 68 years of age who died of carcinoma and at autopsy both coronary arteries were found to be obliterated in their proximal portions. The obliteration was of a character judged to be of long duration. The existence of extensive pericardial adhesions was thought by him to have provided an adequate collateral circulation through the vessels of the parietal pericardium.

Four hearts with partial or complete pericardial obliteration by fibrous adhesions were injected by way of the coronary arteries in the manner described in the previous paper.

*Case 1 (3955).*—A white man, 54 years of age, died of carcinoma of the stomach. At autopsy the pericardium was found to be completely obliterated by fibrous adhesions and the parietal pericardium was adherent bilaterally to the pleurae, the surfaces of which were also fused locally by fibrous adhesions. The heart was not enlarged.

In addition to the coronaries, the vessels of the parietal pericardium were filled by injection mass, and the adhesions between the pericardial surfaces were so extensively injected as to appear almost uniformly black.

Microscopic examination of sections including the myocardium and attached parietal pericardium, showed the intervening tissue to be richly vascularized with vessels filled by injection mass.

The coronary arteries were patent throughout and were the seat of only mild intimal sclerosis.

*Case 2 (3884).*—A white man, 40 years of age, died of gradually progressive cardiac failure due to rheumatic heart disease. At autopsy, rheumatic pancarditis was found, with chronic mitral, aortic and tricuspid valvulitis, cardiac hypertrophy and dilatation, and extensive pericardial adhesions over the anterior surface of the heart. There was a complete injection of the vessels of the parietal pericardium which were especially numerous at the site of the adhesions. Injection mass was seen in the vessels of the diaphragm and of the trachea and bronchi. The coronary arteries were free from sclerosis.

Microscopic examination of sections through the pericardial adhesions disclosed many vessels extending from epicardium to parietal pericardium, filled with injection mass.

*Case 3 (3883).*—A white woman, 50 years of age, died of congestive heart failure due to rheumatic heart disease. At autopsy rheumatic pancarditis was found with chronic mitral, aortic and tricuspid valvulitis, cardiac hypertrophy and dilatation,

and complete obliteration of the pericardial sac by fibrous adhesions. There were scattered pleural adhesions over both lower lobes. The coronary arteries were the seat of only mild intimal sclerosis.

The parietal pericardium was richly vascularized and the vessels were filled with injection mass. The adhesions between pericardial surfaces were rendered black by injection of their vessels.

Microscopic examination of sections, including the adherent pericardial surfaces, disclosed many vessels filled with injection mass. These vessels extended from the remnants of the subepicardial fat out into the parietal pericardium. They were not limited to areas usually the site of subepicardial fat deposits, but were seen bridging the obliterated pericardial space in sections taken from many different portions of the heart.

*Case 4 (4000 P).*—A white man, 34 years of age, died of tuberculous meningo-encephalitis. At autopsy the pericardial sac was found to be completely obliterated by richly vascularized granulation tissue in which there was a thick dissemination of miliary and small conglomerate tubercles. The adhesions between heart and diaphragm were particularly dense and the entire under surface of the diaphragm was covered by tuberculous granulation tissue. The heart was moderately enlarged. The coronary vessels were normal.

Before the coronary arteries were injected all of the peri- and intervascular reflections of the parietal pericardium were cut. In addition, all of the great vessels were cut at the ostia so that the normal sites of anastomoses between cardiac and extracardiac vessels were destroyed. On injection of the coronary arteries, however, there was extensive injection of the vessels of the parietal pericardium with filling of the main trunks of the pericardiacophrenic arteries. The injection of the phrenic arteries was especially marked and the vascularized granulation tissue on the inferior surface of the diaphragm became almost uniformly black. The presence of intravascular injection mass was verified by microscopic examination.

#### SUMMARY

The examination of four hearts, with partial or complete obliteration of the pericardial sac by fibrous adhesions, after injection of the coronary arteries with a colloidal suspension of lamp black showed that the extracardiac anastomoses of the coronary arteries were increased owing to the presence of adhesions. In all four instances a particularly rich injection of the parietal pericardium was obtained and microscopic examination of the adhesions showed them to contain injected vessels, extending from epicardium to parietal pericardium. A microscopic study of cleared blocks (3 mm. in thickness) of myocardium and attached pericardial adhesions, showed the arborization



and anastomosis of branches of the arteries of the parietal pericardium with those of the heart. This vascularization was not limited to the usual areas of subepicardial fat, but was seen in regions not ordinarily containing arterial branches. In no one of the four cases were the coronary arteries significantly diseased.

In one of the four cases, the normal sites of anastomoses between the cardiac and extracardiac vessels were destroyed by cutting away the great vessels entering and leaving the heart, as well as the peri- and intervacular reflections of parietal pericardium. Injection mass was found however, in the arteries of the parietal pericardium and the diaphragm, showing that it has passed directly through the adhesions from coronary to extracardiac vessels.

If the extracardiac anastomoses of the coronary arteries constitute a significant reserve for cardiac circulation, it would appear that this reserve would be augmented by the presence of pericardial adhesions. Direct communication between branches of the coronary arteries and the pericardial branches of the internal mammary arteries with free anastomosis with the anterior branches of the thoracic aorta is established over areas corresponding to the extent of the adhesions. Work is now in progress in this laboratory to study the functional significance of such an experimentally induced collateral circulation in experimental coronary occlusion.

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## EXPLANATION OF PLATE 54

FIGS. 1, 2 and 3. Three photographs of the same block of myocardium with attached fused visceral and parietal pericardium. The coronary arteries were injected with a suspension of lamp black, and the injection mass passed between epicardium and parietal pericardium through the vascularized adhesions. (Case 3.)

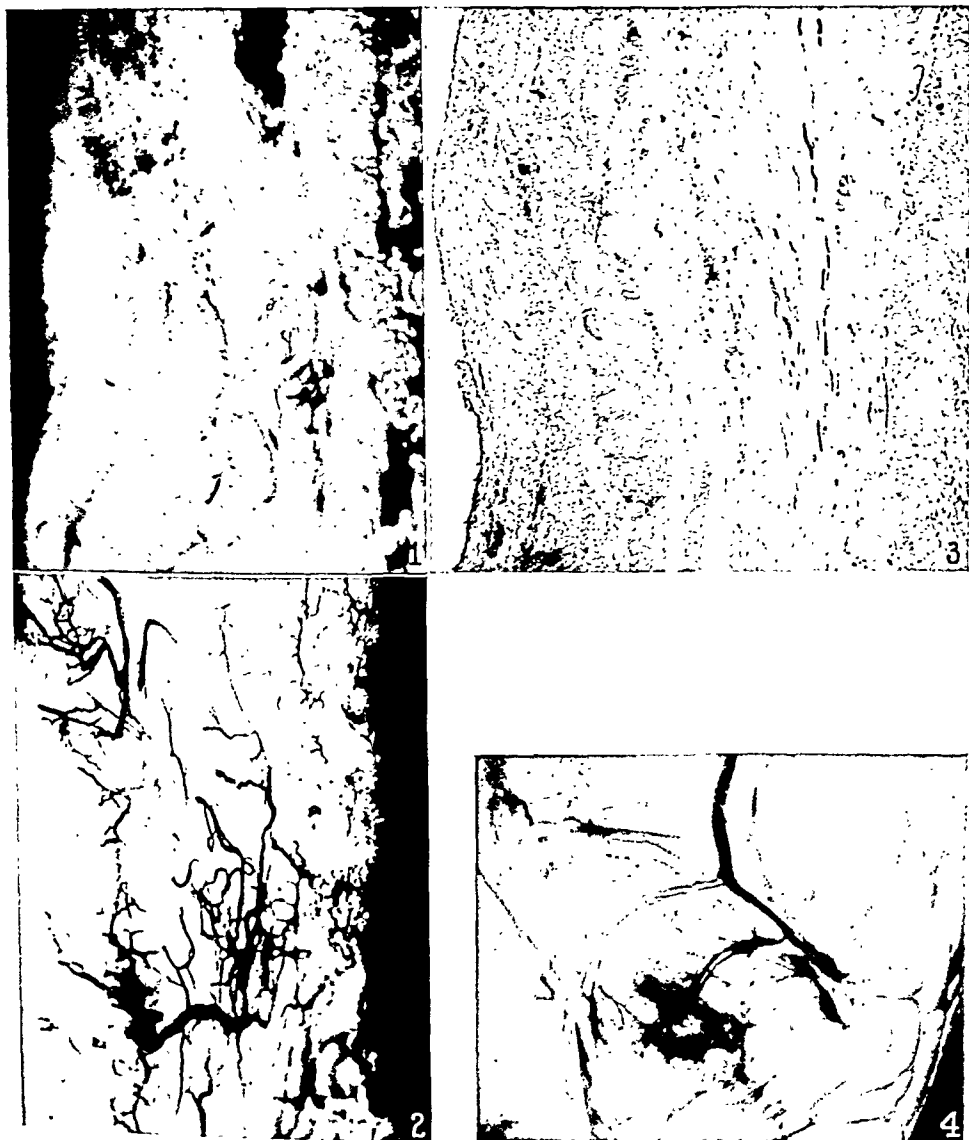
FIG. 1. Formalin-fixed block.  $\times 7$ .

FIG. 2. Cleared block. The anastomoses between vessels of parietal pericardium and epicardium are seen.  $\times 7$ .

FIG. 3. Microphotograph of section from same block stained by hematoxylin and eosin. The adhesions are dense and fibrous and there is considerable indurated fat adherent to the outer surface of the parietal pericardium. The injection mass has dropped out of some of the larger vessels.  $\times 10$ .

FIG. 4. Photograph of parietal pericardium including injected pericardiophrenic artery (Case 4). The great vessels were cut at their ostia and the natural reflections of the parietal pericardium were all cut. The pericardial vessels were injected through the vascularized adhesions from the coronary arteries. Three-fourths natural size.





(Moritz *et al.*: Anastomoses of the coronary arteries)



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